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**14. ABSTRACT**

**Background:** Epithelial to mesenchymal transition (EMT) is a well-established process during embryonic development and cancer progression. Our studies show that a particular microRNA, miR-200c maintains epithelial cell identity. **Hypothesis:** We hypothesize that reintroduction of miR-200c to aggressive breast cancer cells in vivo will restore the epithelial phenotype and thereby render them less aggressive. **Results:** We have identified novel mechanisms by which miR-200c represses neuronal and mesenchymal genes that should not be expressed in epithelial cells. Such genes are inappropriately allowed to be translated in TNBC lines that have lost miR-200c. We have identified targets of miR-200c that mediate cell migration. We show that these are direct targets and that when miR-200c is restored to TNBC cells, migration is decreased because miR-200c targets the genes encoding fibronectin and moesin. We find that restoration of miR- 200c to TNBC cells renders there morphology in 3D culture smaller and rounder as opposed to their usual stellate, invasive structures. We also demonstrate that miR-200c can reverse anoikis resistance in TNBC and we identified TrkB and its ligand NTF3as the direct target that mediates this effect. We find that restoration of miR-200c represses the ability to migrate and invade, and to resist anoikis and chemotherapy, all important steps in the metastatic cascade. Thus, restoration of miR-200c has exciting potential therapeutic value, but further in vivo studies are necessary as proof of principal for feasibility and practicality of non-viral mediated delivery.

**15. SUBJECT TERMS**
microRNA-200c, EMT, breast cancer, invasion

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**Final Progress Report for MicroRNA-200c: a novel way to attack breast cancer metastases by restoring the epithelial phenotype.**
**Therapies:**

## Abstract

**Background:** Epithelial to mesenchymal transition (EMT) is a well-established process during embryonic development, allowing for the migration of cells and groups of cells in developing tissues. EMT in adult tissues is recognized as a molecular mechanism utilized by cancer cells during the process of tumor progression. During EMT, epithelial cells actively down regulate cell-cell adhesion systems, lose polarity, and acquire mesenchymal markers and migratory capability, resulting in reduced intercellular interactions and increased invasive capacity. Transcription factors such as Twist, Snail, Slug, SIP1 (ZEB2), and ZEB1, have been shown to control EMT by repressing E-cadherin (expression of which is a hallmark characteristic of epithelialness). We find that ZEB1, normally only expressed in mesenchymal cells and not in breast epithelial cells, is expressed in aggressive breast cancers that have undergone an EMT. Our studies show that a particular microRNA, miR-200c, directly targets ZEB1 and is responsible for maintaining epithelial cell identity. Our preliminary data show that in a panel of breast cancer cell lines, those that retain E-cadherin and lack ZEB have ~100 fold higher miR-200c expression as compared to cell lines which express high ZEB1 and have lost E-cadherin (a hallmark of EMT) and gained mesenchymal markers. **Hypothesis:** We hypothesize that reintroduction of miR-200c to aggressive breast cancer cells *in vivo* will restore the epithelial phenotype and thereby render them less invasive or cause regression of established metastases, or at least restore chemo-sensitivity. If our hypotheses prove true, miR-200c has promising potential as a new therapeutic agent. **Specific Aims: Aim 1 –** To determine the mechanisms by which miR-200c serves to maintain the epithelial nature of breast cancer cells and how its loss leads to epithelial to mesenchymal transition (EMT). **Aim 2 –** To examine miR-200c in normal breast and clinical specimens of breast cancers with varying metastatic potential. **Aim 3 –** To perform *in vivo* proof of principal experiments to determine if restoration of miR-200c levels will render invasive breast cancer cells less invasive or if miR-200c can cause regression of already established metastases. **Study design:** We will use a combination of breast cancer cell lines, archival patient samples, and *in vivo* mouse models of metastasis to determine how and why miR-200c is so critical for maintaining proper epithelial cell identity and how it is lost in breast cancer cells that undergo EMT. Most importantly, our *in vivo* studies will utilize models of metastasis as proof of principal to determine if re-introduction of miRNA-200c can prevent or reverse breast cancer metastasis. These types of studies will directly evaluate the therapeutic value of miRNAs. Thus, our proposal is **innovative** because it uses creative ways to evaluate, *in vivo*, the potential of miRNAs to serve as a form of differentiation therapy. It will also seek to determine the ways, other than repression of ZEB1, that miR-200c maintains the epithelial phenotype and how loss of miR-200c occurs in aggressive breast cancers. These studies have high **impact** and potential to improve treatment of patients, because it is metastatic disease that causes breast cancer mortality and if we could find an effective way to prevent or treat metastatic breast cancer it would save many lives. This type of "differentiation therapy" should cause cancer cells to revert back to a more normal state and thus it should have few toxic side effects. The proposed studies are the first steps towards using microRNA-200c as a novel

therapeutic to prevent or treat metastatic breast cancer and if these pre-clinical studies work, it should not take long to move this type of therapy to the clinical trial setting.

**Introduction:** In this research study we will characterize the role of the microRNA, miR-200c, in breast cancer metastasis. We have found that breast cancer cells that retain E-cadherin and estrogen receptors (ER) have high levels of miR-200c and therefore express no ZEB1. In contrast the more de-differentiated and more aggressive cell lines that have lost E-cadherin and ER have low miRNA200c and thus have high levels of ZEB1, which we and others have shown is a master repressor of "epithelialness." Our preliminary data show that miR-200c is a potent mediator of EMT. Reintroduction of miR-200c results in restoration of an epithelial phenotype (as initially judged by re-expression of E-cadherin), reduced invasiveness and increased sensitivity to chemotherapeutic drugs. We will use *in vivo* preclinical models as proof of principal to test whether restoration of miR-200c has potential as a therapeutic strategy for the treatment of breast cancer. These studies have high potential to improve treatment of patients, because it is metastatic disease that causes breast cancer mortality. We put forth the interesting and novel hypothesis that a miRNA normally represses expression of genes that can render cancer cells capable of metastasizing and that it may be a useful biomarker and potential therapeutic target for invasive disease. This strategy will provide the test the feasibility of using miR-200c as a new therapeutic agent for preventing breast cancer progression.

**BODY:**

**Statement of Work**

**Aim 1 – To determine the mechanisms by which miR-200c serves to maintain the epithelial nature of breast cancer cells and how its loss leads to epithelial to mesenchymal transition (EMT).**

**a. We will confirm that restoration of miR200c reduces migration and invasion capacity and renders them more sensitive to chemotherapeutic agents. These studies will be performed in the first 6 months of the first year.**

**b. To determine if miR-200c affects sensitivity to paclitaxel.** 4d to detect any significant affect of miR-200c on chemosensitivity.

**c. To determine other mechanisms by which miR-200c serves to maintain the epithelial nature of breast cancer cells.**

**Summary of Aim 1 results**:
Previous results regarding Aim 1a were reported in two manuscripts (and prior progress reports) demonstrating that restoration of miR-200c to triple negative breast cancer cells that have lost miR-200c results in significantly (~80%) reduction in their ability to migrate and invade and that this was at least partially due to miR-200c targeting ZEB1 and thereby allowing restoration of E-cadherin, which holds cells together [1, 2]. However, we realized that even in some cells in which E-cadherin did not come back on likely because of silencing by methylation, miR-200c still caused a reduction in the

amount of migration and invasion. We therefore followed up on other targets that were identified in the original screen [2] that we thought might have something to do with the ability of miR-200c to repress the ability to actively move because in order to move cells not only need to lose attachment to their neighbors, but they need to activate components of the cytoskeleton that allow them to actively move in a directional manner. Indeed we found that genes encoding fibronectin 1 (FN1) and moesin (MSN) were directly targeted by miR-200c causing repression of these genes (that should not be on in normal epithelial cells) and that shutting down these genes via restoration of miR-200c was responsible for the reduction in migration observed upon restoration of miR-200c and we published this finding during this last year [3]. This paper was highlighted in the journal Breast Cancer Research for its high impact findings.

Regarding Aim 1b: We previously demonstrated that miR-200c restores chemosensitivity to taxanes in endometrial cancer and ovarian cancer cell lines by reducing expression of its direct target class III beta tubulin (TUBB3) [1, 4]. However, despite the fact that TUBB3 is known to be overexpressed in clinical breast cancer samples [5-13], we are having difficulty finding a cell line that models this. The MDA-MB-231 are resistant to taxanes because they have a mutated class 1 beta tubulin [14]. We found 3 other triple negative cell lines that are taxane resistant that overexpress TUBB3 (HCC70, BT549, and DU4475) (Supporting Data Figure 3 in last progress report). However, the BT549 express so much TUBB3 that the miR-200c mimic did not repress it and the other two have have high miR-200c already and for some reason it appears not to be functional because it is failing to repress miR-200c. We could sequence the TUBB3 3'UTR in these cells and determine if the miR-200c binding site is mutated such that miR-200c can no longer bind to and repress TUBB3 expression; however we have not yet done so. We are currently strying to find a breast cancer line or primary tumors that a colleague is growing as explants that are resistant to taxanes by this method (overexpression of TUBB3 as a result of loss of miR-200c), however in the future it may be more worthwhile to actually sequence TUBB3 in clinical samples known to be resistant to taxanes and determine if they have lost the TUBB3 binding site or if the 3'UTR is truncated such that this site is lost if miR-200c is still expressed. This remains an open avenue that needs to be further pursued.

Regarding Aim 1c: to determine other mechanisms by which miR-200c serves to maintain the epithelial nature of breast cancer cells, we have thoroughly addressed this aim. Although others have also studied various aspects miR-200c serves as a guardian of the epithelial phenotype, we have contributed substantially to the body of literature on this topic [1-3, 15-17] which includes two invited reviews on the topic [15, 17] and invited talks for Dr. Richer and graduate student Erin Howe. We noted early on that restoration of miR-200c to triple negative breast cancers results in repression of a whole program of mesenchymal and neuronal genes that should not be expressed in normal epithelial cells, but that are expressed in triple negative breast cancers [2, 3]. Graduate student Erin Howe, who was supported by this grant decided to focus on genes that we identified as being expressed in TNBC that were associated with the ability to resist anoikis (death by detachment) because this ability is thought to be necessary for invasion through stroma and survival in blood or lymphatic vessels. She noted that one of the genes that was repressed when she restored miR-200c to TNBCs is TrkB, a neuronal tyrosine kinase receptor that had been identified in a genome-wide screen for genes that caused anoikis resistance in normal gut intestinal epithelial cells [18]. Indeed, TrkB, and its primary ligand brain-derived neurotrophic factor (BDNF) induce anoikis resistance in a variety of carcinoma models including breast [19], ovarian [20-22], and head and neck [23]. This past year my student Erin Howe published in Breast Cancer Research that TrK is directly targeted by miR-200c [3] As noted above, this paper [3] was very well-received and highlighted because of its high impact in the field [24]. Indeed Erin just received notification that her pre-doctoral NRSA grant centered around these findings was funded through the NCI. In an additional manuscript in preparation she finds that anoikis resistance does indeed correlate with loss of miR-200c in breast cancer cell lines and restoring miR-200c renders them sensitive to anoikis (Figure 1). She demonstrates that putting in exogenous TrkB is not enough to induce anoikis resistance in luminal A breast cancer cell lines, but one of its ligands (either BDNF or neurotrophin 3 (NTF3)) is required as well (Fig 2). This suggests that anoikis resistance requires an inappropriate receptor/ligand signaling loop.

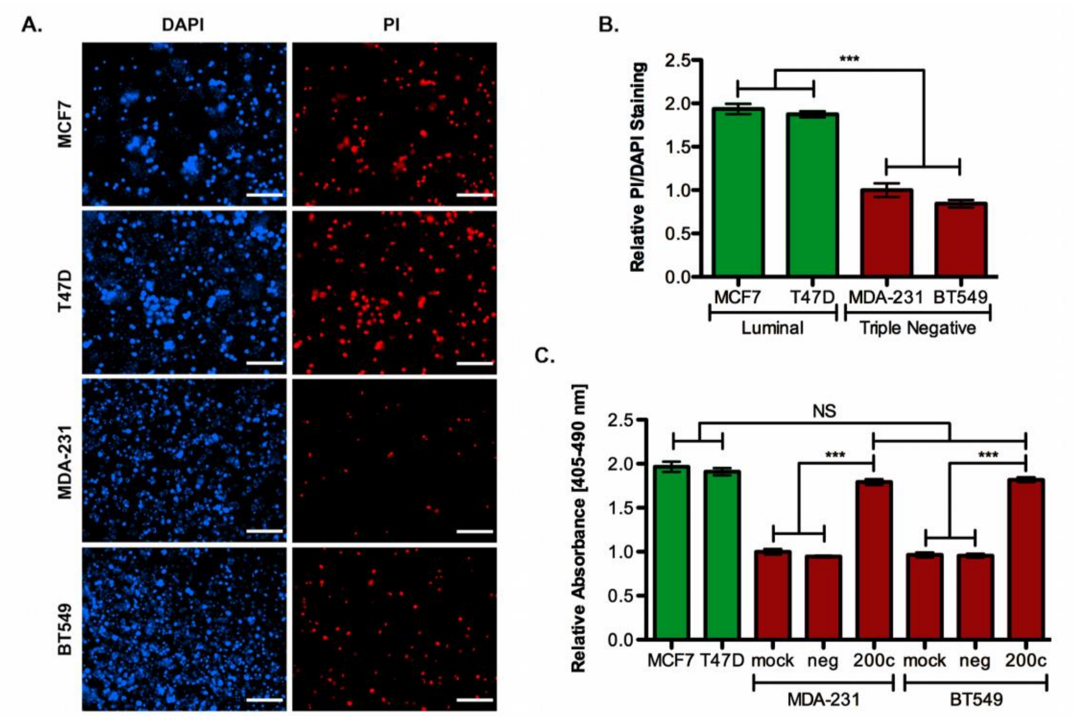



***Figure 1. Triple negative breast cancer cells are more anoikis resistant than luminal cells and miR-200c sensitizes aggressive cells to anoikis.***

***A.*** *Cells were plated attached or suspended for 24 hrs prior to staining with DAPI and propidium iodide (PI). Representative images of suspended cells are shown, scale bar 50 μm.* ***B.*** *Quantitation of data in A, presented as a ratio of PI to DAPI, with each cell line normalized to the attached condition. Shown relative to MDA-231 cell line. Columns, mean of three biological replicates, bars, standard error of the mean. ANOVA with Tukey post-hoc test, *** $p < 0.001$.* ***C.*** *Cells treated with transfection reagent only (mock), scrambled negative control (neg) or miR-200c mimic (200c) and 24 hrs later plated in suspension. 24 hrs post suspension, a cell death ELISA was performed. Data normalized to attached condition and shown relative to MDA-231 mock transfection. Columns, mean of three biological replicates, bars, standard error of the mean. ANOVA with Tukey post-hoc test, *** $p < 0.001$, NS – not significant.*

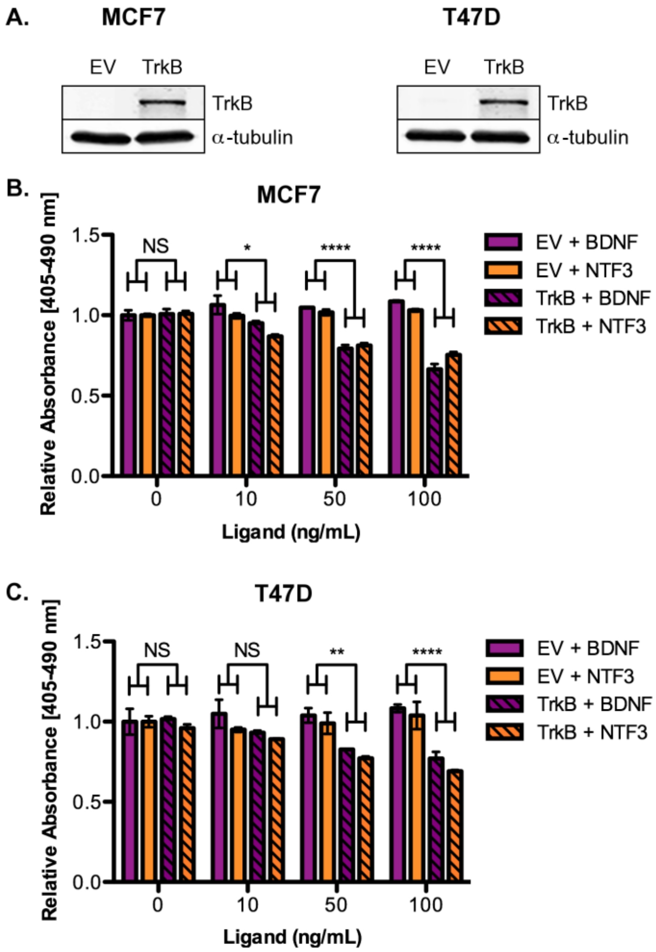



***Figure 2. TrkB requires ligand to induce anoikis resistance. A.*** *MCF7 and T47D cells were stably selected for expression of empty vector (EV) or TrkB. Immunoblot showing TrkB expression, α-tubulin used as loading control. MCF7,* ***B****, and T47D,* ***C****, cells were plated suspended in increasing concentrations of BDNF or NTF3. Cells were harvested 24 hrs later and apoptosis assayed by cell death ELISA, data normalized to attached condition and shown relative to EV conditions. Columns, mean of three biological replicates, bars, standard error of the mean. Two-way ANOVA, Bonferroni multiple comparison, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.*

In the manuscript in preparation, Erin additionally demonstrates that not only is the TrkB receptor directly targeted by miR-200c, but the ligand NTF3 is also a direct target of miR-200c and loss of miR-200c. Thus loss of miR-200c in TNBC removes the repression of translation of this inappropriate receptor/ligand signaling loop.

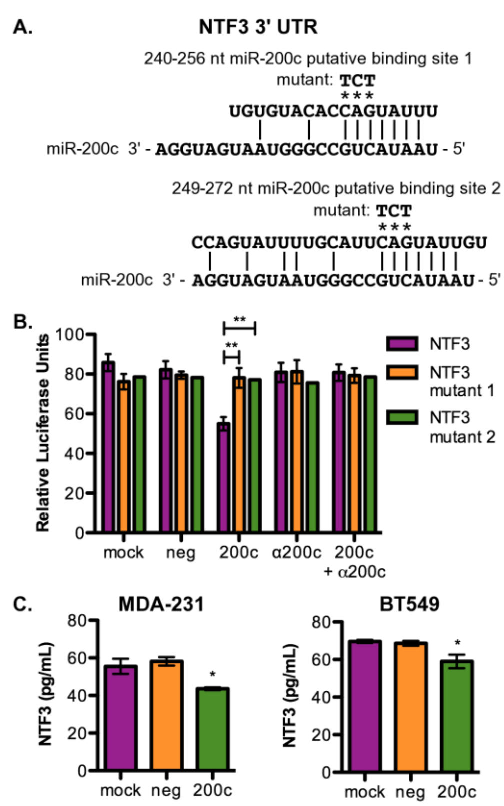



***Figure 3****. NTF3 is a direct target of miR-200c.* ***A****. Regions of the 3' UTR where miR-200c is predicted to bind.* ***B.*** *Hec50 cells treated with transfection reagent only (mock), scrambled negative control (neg), miR-200c mimic (200c), miR-200c antagomiR alone (α200c) or in conjunction with miR-200c (α200c + 200c) and luciferase assay performed. Columns, mean of five biological replicates, bars, standard deviation of the mean. Two-way ANOVA, Bonferroni multiple comparison, ** $p < 0.01$.* ***C****. Cells transfected with miRNA constructs and 48 hrs later medium collected for analysis by NTF3 ELISA. Columns, mean of three biological replicates, bars, standard error of the mean. ANOVA, * $p < 0.05$.*

She then found that the mechanism by which this receptor/ligand autocrine loop transmits a survival signal to facilitate anoikis resistance in breast cancer cells is via Akt signaling (Fig 4).

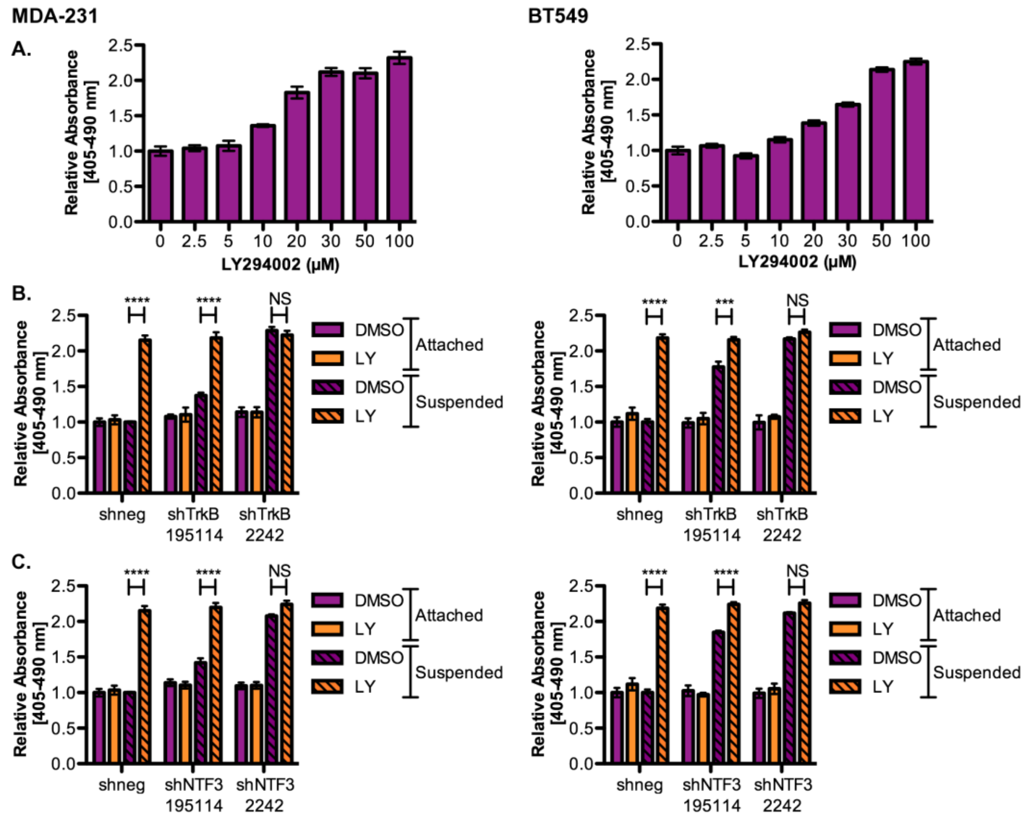



***Figure 4. Akt signaling is downstream of TrKB/NTF3 in anoikis resistant cells*** *__A.__ Cells were plated in suspension in increasing concentrations of LY294002. Cells were harvested 24 hrs later and apoptosis assayed by Cell Death ELISA, data presented relative to 0 µM condition. Columns, mean of three biological replicates, bars, standard error of the mean. shTrkB, __B__, and shNTF3, __C__, cells were plated in suspension in 50 µM LY294002 and assayed as in A, data presented relative to shneg vehicle treated cells. Columns, mean of three biological replicates, bars, standard error of the mean. Two-way ANOVA, Bonferroni multiple comparison, *** p < 0.001, **** p < 0.0001, NS – not significant.*

Interestingly, although loss of miR-200c would allow TrkB and NTF3 to be translated in TNBC lines, we speculated that something had to drive transcription of these genes in the suspended cells (Fig 5)

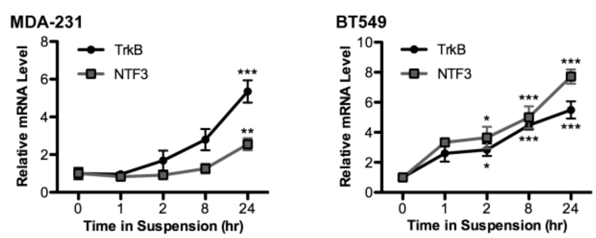
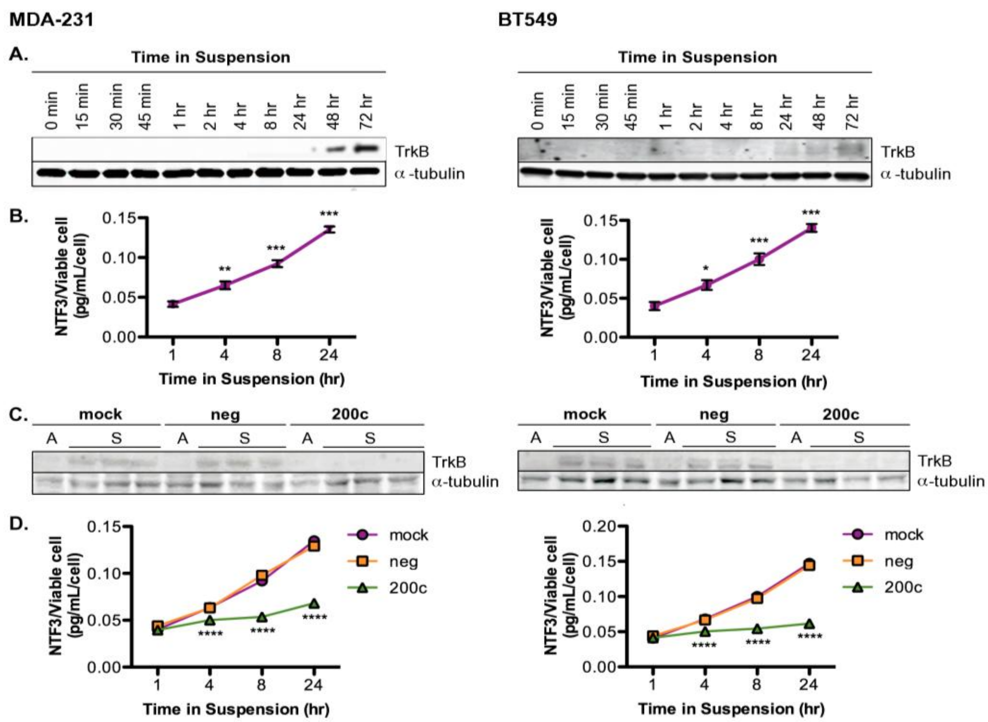



***Figure 5. TrkB and NTF3 up-regulation is transcriptional****Cells were plated in suspension and RNA was harvested at time points indicated. SYBR green qRT-PCR was performed for TrkB and NTF3. Data normalized to actin and presented relative to attached time point. Points, mean of three biological replicates, bars, standard error of the mean. ANOVA, Dunnett multiple comparison, * p < 0.05, ** p < 0.01, *** p < 0.001.*

Interestingly, TrkB protein and the NTF3 ligand secreted into the media both increase upon forced detachment (Fig 6).

***Figure 6. TrkB and NTF3 are up-regulated in suspended cells and miR-200c blocks this up-regulation****. Cells were plated in suspension and harvested at the time points indicated. __A.__*

*Immunoblot for TrkB expression, α-tubulin used as loading control.* ***B.*** *NTF3 ELISA performed on medium. Points, mean of three biological replicates, bars, standard error of the mean. ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Cells treated with transfection reagent only (mock), scrambled negative control (neg) or miR-200c mimic (200c) and 24 hrs later plated in suspension.* ***C.*** *Cells were harvested 24 hrs later and immunoblot performed for TrkB, α-tubulin used as loading control.* ***D.*** *NTF3 ELISA performed on medium at time points indicated. Points, mean of three biological replicates, bars, standard error of the mean. Two-way ANOVA, Bonferroni multiple comparison, **** $p < 0.0001$.*




Consequently, the question to be ask next is what is driving the expression of this receptor ligand pair at the transcriptional level? Erin looked at predicted binding sites in the promoters of the genes encoding both receptor and ligand. She determined that both contained NF-κB binding sites. In figure 7 she demonstrates that indeed NF-κB transcriptional activity increases in TNBC cells when they are grown in forced suspension.

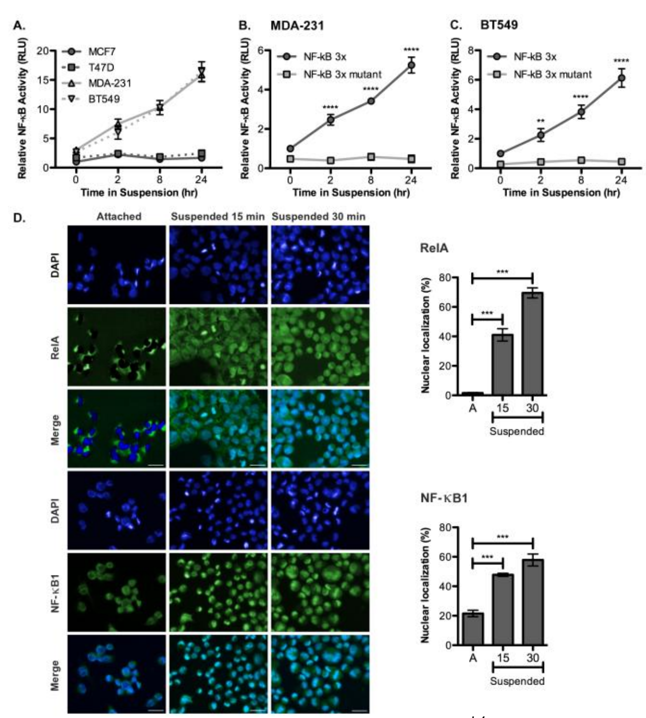



***Figure 7. NF-κB transcriptional activity increases in suspended TNBC cells A.*** *Cells were transfected with 3x NF-κB transcriptional response element reporter and a Renilla control and 24 hrs later plated in suspension. Cells were harvested at time points indicated and dual luciferase assay performed. Data normalized to attached time point and presented relative to MCF7 attached condition. Columns, mean of three biological replicates, bars, standard error of the mean. MDA-231,* ***B****, and BT549,* ***C****, cells were transfected with 3x NF-κB or mutant reporter and assayed as in A. Data presented relative to NF-κB attached condition. Columns, mean of three biological replicates, bars, standard error of the mean. Two-way*

*ANOVA, Bonferroni multiple comparison, ** p < 0.01, **** p < 0.0001.* ***D.*** *BT549 cells were grown on coverslips (attached), or in suspension and spun onto slides. Immunocytochemistry was performed for RelA or NF-κB1 (left), and the percentage of nuclear staining at each time point was quantitated (right). Columns, mean of three biological replicates, bars, standard error of the mean. ANOVA with Tukey post-hoc test, *** p < 0.001.*

Lastly for this manuscript we demonstrate  by chromatin immunoprecipitaton analysis (ChIP) that NF-κB directly binds to specific sites in the promoters of TrkB and NTF3 in suspended cells to upregulate these genes.

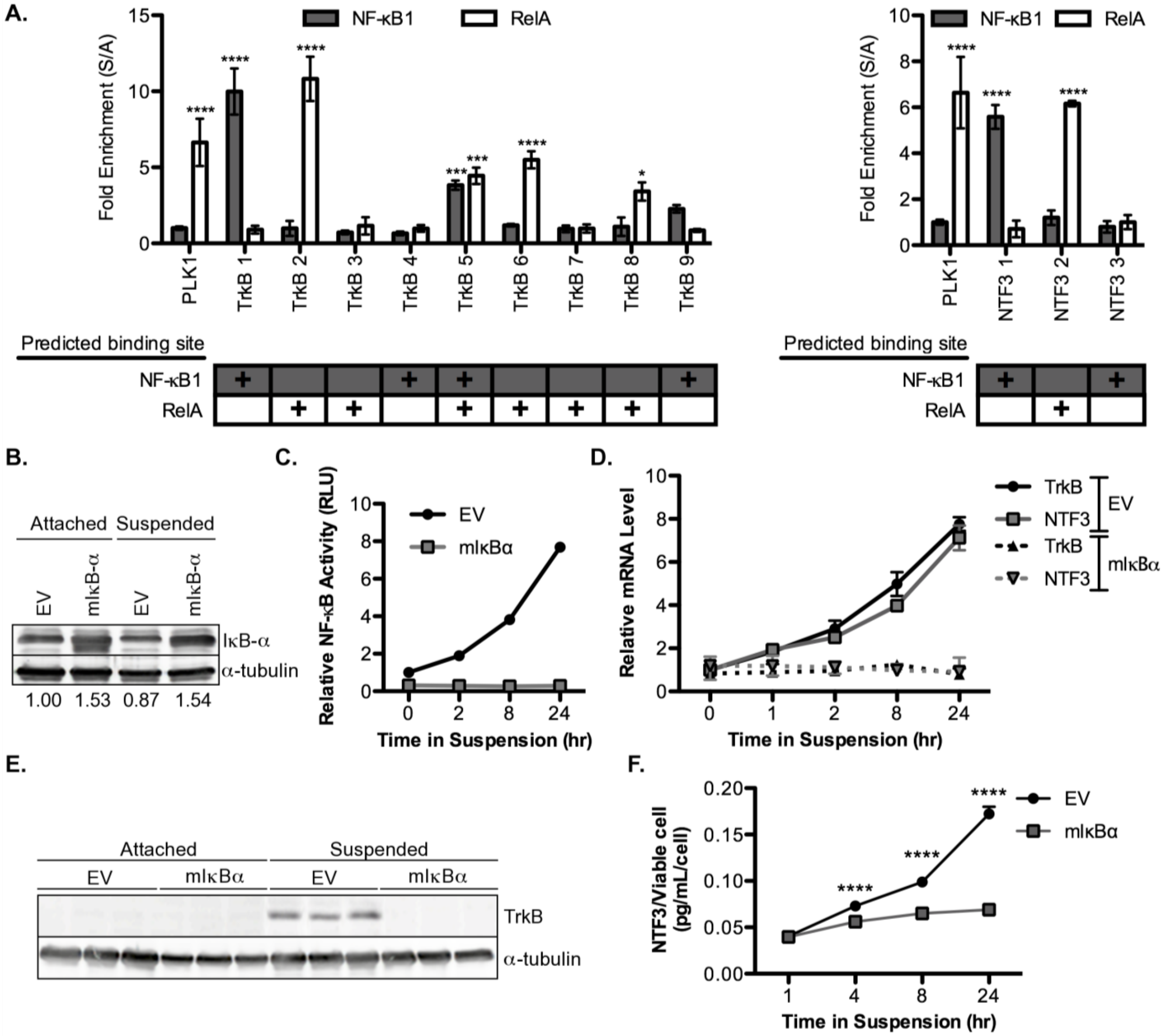
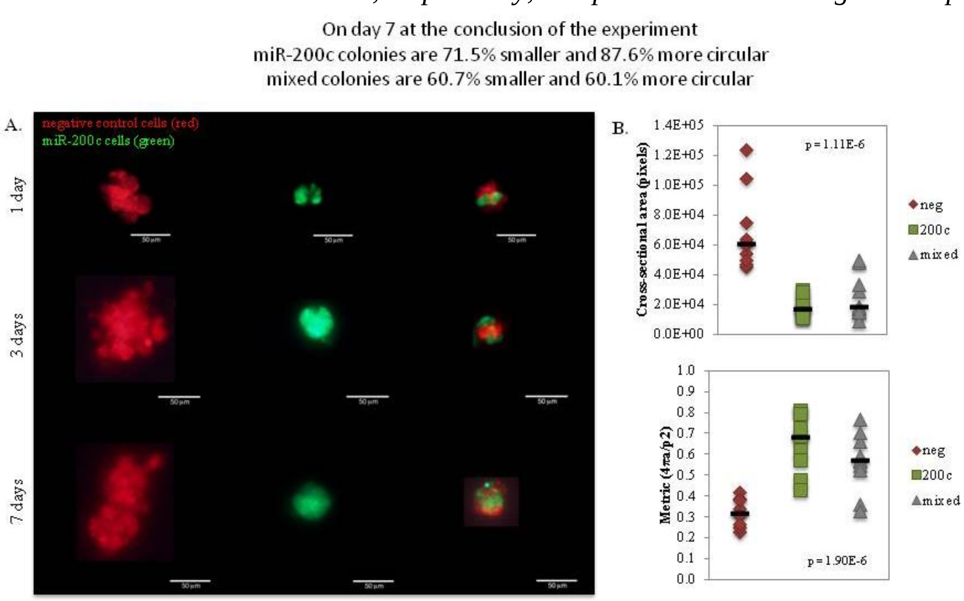



***Figure 8. NF-κB directly binds to the promoters of  TrkB and NTF3 in suspended cells to upregulate these genes. A.*** *BT549 cells were plated in suspension for 2 hrs and harvested for ChIP analysis. Following precipitation with antibodies against NF-κB1 and RelA, SYBR green qRT-PCR was performed for sites in the TrkB (left) and NTF3 (right) promoters. PLK1 used as a*

*positive control for increased RelA binding in suspended cells. Data was normalized to input controls and presented as a ratio of suspended over attached conditions. Columns, mean of three biological replicates, bars, standard error of the mean. Student's t-test, * p < 0.05, *** p < 0.001, **** p < 0.0001.* ***B-E*** *BT549 cells stably selected for empty vector (EV) or genetic NF-κB inhibition through mutant IκBα (mIκBα).* ***B.*** *Characterization of mIκBα cells, immunoblot of IκBα, α-tubulin used as loading control.* ***C.*** *Cells were transfected with 3x NF-κB transcriptional response element reporter and a Renilla control and 24 hrs later plated in suspension. Cells were harvested at time points indicated and dual luciferase assay performed. Data normalized to attached time point and presented relative to EV condition. Points, mean of three biological replicates, bars, standard error of the mean.* ***D.*** *Cells were plated in suspension and RNA was harvested at time points indicated. SYBR green qRT-PCR was performed for TrkB and NTF3. Data normalized to actin and presented relative to attached. Points, mean of three biological replicates, bars, standard error of the mean.* ***E.*** *Cells were plated in suspension for 24 hrs and harvested for immunoblot analysis of TrkB, α-tubulin used as loading control.* ***F.*** *NTF3 ELISA performed on medium at time points indicated. Columns, mean of three biological replicates, bars, standard error of the mean. Two-way ANOVA, Bonferroni multiple comparison, **** p < 0.0001.*

Since miR-200c represses ZEB1, which is a transcriptional repressor known to repress other genes involved in polarity in addition to E-cadherin, we sought to determine if miR-200c is able to restore polarity to TNBC cells in 3-D culture. To determine if miR-200c can restore polarity to aggressive breast cancer cells, we used MDA-231 and BT549 cells stably expressing DsRed or ZsGreen transfected with either scrambled negative control or miR-200c mimic, respectively, and plated in growth factor reduced Matrigel. Although this work is still in progress, because we are paraffin embedding and staining for various polarity marker, the results are promising in that the colonies are much more round and smaller and interestingly, cells that got miR-200c force cells that did not to take on the smaller, rounder configuration of the whole colony and this held true in two TNBC cell lines, the MDA-MB-231 (Fig 9) and the BT549 (Fig 10) and these data are analyzed quantitatively in Figure 11.

***Figure 9: miR-200c decreases size and increases circularity of MDA-231 cells in 3-D culture.*** *MDA-231 cells stably expressing DsRed or ZsGreen were transfected with a scrambled negative control or miR-200c mimic, respectively, and plated in GFR Matrigel. 10 representative images were taken for each condition negative control (neg), miR-200c (200c) or colonies containing negative and miR-200c cells (mixed) at each time point. A. Representative images for each condition at each time point. B. (Top) The cross-sectional area was determined*



*for each colony. (Bottom) Metric* showing how closely the colony approximates a circle with 1 being a perfect *circle. Points, each colony, lines, mean colony size. p determined by ANOVA.*

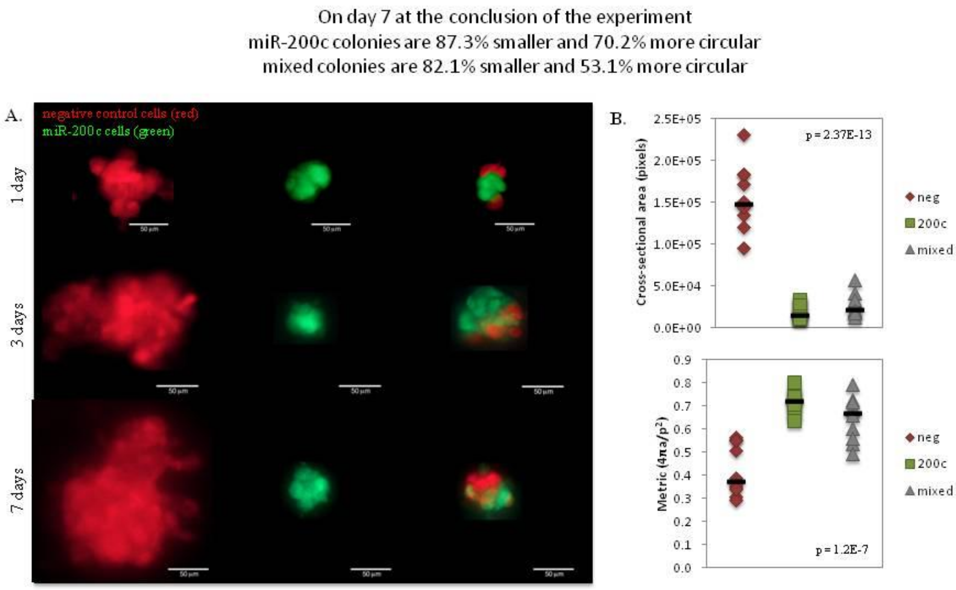



***Figure 10. miR-200c decreases size and increases circularity of BT549 cells in 3-D culture.*** *BT549 cells stably expressing DsRed or ZsGreen were transfected with a scrambled negative control or miR-200c mimic, respectively, and plated in GFR Matrigel. 10 representative images were taken for each condition negative control (neg), miR-200c (200c) or colonies containing negative and miR-200c cells (mixed) at each time point. A. Representative images for each condition at each time point. B. (Top) The cross-sectional area was determined for each colony. (Bottom) Metric showing how closely the colony approximates a circle with 1 being a perfect circle. Points, each colony, lines, mean colony size. p determined by ANOVA.*

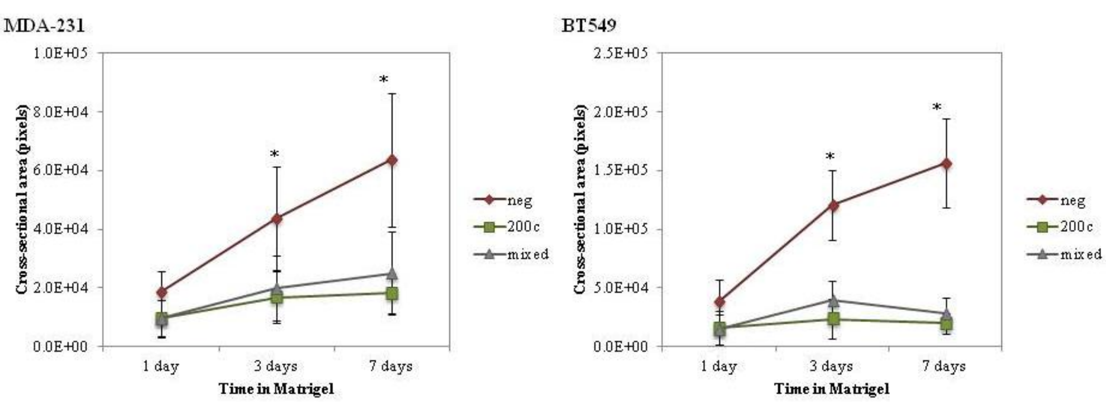



***Figure 11: miR-200c and mixed colonies growth arrest in 3-D culture.*** *Cells stably expressing DsRed or ZsGreen were transfected with a scrambled negative control or miR-200c mimic, respectively, and plated in GFR Matrigel. 10 representative images were taken for each condition negative control (neg), miR-200c (200c) or colonies containing negative and miR-200c cells (mixed) at each time point. The cross-sectional area was determined for each colony. Points, mean of 10 colonies, bars, standard deviation of the mean. * p < 1.0E-4, ANOVA.*

**Aim 2 – To examine miR-200c in normal breast and clinical specimens of breast cancers with varying metastatic potential and determine the mechanisms whereby miR-200c is lost or suppressed in aggressive breast cancers.**

**a. To examine miR-200c in normal breast and clinical specimens of breast cancers with varying metastatic potential.** We have performed in situ hybridization for numerous other miRNAs on clinical samples with success; however, despite trying several different methods of detection we were not able to get it to work even in paraffin embedded breast cancer cell lines that we know to have high miR-200c levels by RT-PCR. However, we did perform experiments demonstrating that miR-200c (which is high in ERalpha positive breast cancers) positively regulates Dicer, the enzyme responsible for the final step in microRNA biogenesis [16]. We also showed that an miRNA family miR-221/222 that is one of the few that is higher in ER negative breast cancers than ER positive directly targets and represses not only ERalpha but also Dicer itself [16]. We did perform in situ hybridization for miR-222 and find that indeed it is high in TNBC but off in ER+ (luminal A tumors) [16].These findings likely explain why ER positive breast cancers express more miRNAs at higher levels than ER negative (particularly the triple negative breast cancers). We just completed a review with some of this primary data as well about the opposing effects of the miR-200 family versus the miR-221/222 family on epithelial cell identity [15]

**b. Determine the mechanism whereby miR-200c is lost or suppressed in aggressive breast cancers.**

During the time that this grant covered two publications demonstrated that miR-200c is lost by both promoter methylation and by transcriptional repression mediated by ZEB1 [25, 26] We also demonstrated that knocking down ZEB1 with shRNAs increased miR-200c and restored E-cadherin [1]. We did perform some FISH analysis for miR-200c on cell lines that we knew to not express miR-200c and there were not any gross deletions (although we could not rule out microdeletions) (see attached report in appendix). Since these initial studies in cell lines were not indicating that there were deletions and the papers came out on this topic indicating that it was likely epigenetic events rather than deletions that lead to loss of miR-200c in breast cancer, we did not perform the FISH analysis on clinical samples. However, it remains to be shown shown on a large scale in clinical breast cancer samples, that epigenetic silencing by methylation is the only method by which miR-200c is shut down versus microdeletions of the chromosomal region involved.

**Task 3. Aim 3: To determine the effects of restoration of miR-200c levels on tumor metastasis using two *in vivo* models of metastasis.**

a. **To determine if restoration of miR-200c by intranasal delivery will cause regression of already established lung metastases.** We will use 10 mice per group (miR-200c or a scrambled control microRNA in the same vector; 20 mice total).
b. **To determine if restoration of miR-200c will inhibit metastasis or if it can cause regression of already established metastatic disease, we will use a spontaneous orthotopic xenograft model of breast cancer**. All results from the imaging and microscopy will be analyzed by ANOVA. We will use 30 mice (10 per group, with the 3 groups being no doxycycline, plus doxycyline with cells that

will turn on miR-200c, plus doxycycline with vector that expresses a scrambled control microRNA and LacZ instead of miR-200c) for each of the two experiments described for a total of 60 mice total in the 2 experiments in Aim3b.

We have submitted a no cost extension to this grant to complete this animal work. We just got approval from our IACUC office (see letter in appendix) and will now submit the protocol to the DOD.

**KEY RESEARCH ACCOMPLISHMENTS:**

**Aim 1**

1. We have identified 18 genes statistically significantly altered upon restoration of miR-200c, which are also putative direct targets of miR-200c. We have experimentally confirmed 5 of these as direct targets using a luciferase reporter vector,pMIR-Report (these were reported in Cochrane et al MOLECULAR CANCER THERAPEUTICS 2009 and previous progress reports.
2. We have confirmed that the message levels of these genes and some of their protein products (in the cases for which antibodies exist) are altered by restoration of miR-200c. (Cochrane et al MOLECULAR CANCER THERAPEUTICS 2009 andSupporting Data Figure 2 in last progress report)
3. We have shown that restoration of miR-200c dramatically reduces invasion in vitro. We have accomplished this in two different ways. The first is by knocking down ZEB1 with lentiviral shRNA, which when completely effective, results in upregulation of endogenous miR-200c (see Figure 1 Cochrane et al J Oncology, 2010). The first utilizes microRNA mimics of miR-200c delivered transiently to MDA-MB-231 breast cancer cells that have lost miR-200c (see Figure 2 and Cochrane et al J Oncology, 2010).
4. We have demonstrated that the ability of miR-200c to decrease invasiveness is independent of its ability to restore E-cadherin. In some cell lines, restoration of miR-200c does restore E-cadherin by virtue of repressing the miR-200c direct target ZEB1, which is a transcriptional repressor of E-cadherin. In contrast, in some cell lines, E-cadherin is not restored (could either be because of chromosomal loss or silencing mutation); however, migration and invasion are still dramatically reduced. We believe that this is therefore must be due to one of the other direct or indirect effects of miR-200c. We have identified numerous other genes that change in response to restoration of miR-200c that are known to control migration and invasion (see Figure 2 supporting data, progress report year 2 and Howe et al Breast Cancer Research 2011).
5. We have demonstrated that restoration of miR-200c to MDA-MB-231 cells reduces adhesion to basement membrane complex and laminin (Figure 3 Cochrane et al J Oncology, 2010).
6. We have definitively shown that miR-200c restores chemosensitivity to taxanes in endometrial cancer and ovarian cancer cell lines by reducing expression of its direct target class III beta tubulin (TUBB3) [1, 4]. However, despite the fact that TUBB3 is known to be overexpressed in clinical breast cancer samples [5-13], we are having difficulty finding a cell line that models this. The MDA-MB-231 are resistant to taxanes because they have a mutated class 1 beta tubulin [14]. We found 3 other triple negative cell lines that are taxane resistant that overexpress

TUBB3 (HCC70, BT549, and DU4475) (Supporting Data Figure 3 in last progress report). The BT549 express so much TUBB3 that the miR-200c mimic did not repress it. We are currently still trying to find a breast cancer line or primary tumors that a colleague is growing as explants that represent a line that is resistant to taxanes by this method (overexpression of TUBB3 as a result of loss of miR-200c).

7. TNBC cells are more anoikis resistant than luminal cells and miR-200c sensitizes aggressive cells to anoikis. Demonstrated that TrkB requires ligand to induce anoikis resistance.
8. Proof that the gene encoding one of the TrkB ligands, NTF3, is a direct target of miR-200c (cloned its 3'UTR downstream of luciferase in a reporter).
9. Provided proof that miR-200c suppresses anoikis resistance through targeting of the TrkB/NTF3 signaling axis.
10. Demonstrated that Akt signaling is the signal by which TrkB/NTF3 transmits a survival signal in anoikis resistant cells.
11. Demonstrated that TrkB and NTF3 are upregulated to facilitate survival in anoikis resistant cells when they are cultured under conditions that force growth in suspension.
12. Proved that NF-κB transcriptional activity increases in suspended TNBC cells and that NF-κB directly drives transcription of TrkB and NTF3 in suspended cells by binding to their promoters and then loss of miR-200c relieves the repression that exists in normal breast epithelium or even well differentiated (ER+) tumors, and inappropriately allows translation of these genes into protein.
13. Demonstrated that miR-200c makes TNBC cells grow in significantly smaller, well rounded colonies.
14. Performed FISH analysis on TNBC cell lines and found that there is not a gross deletion in the chromosomal area in which miR-200c is located.

## REPORTABLE OUTCOMES:

### Manuscripts

Howe EN, Cochrane DR and **Richer JK**. The miR-200 and miR-221/222 miRNA Families: Opposing Effect on Epithelial Identity. *JOURNAL OF MAMMARY GLAND BIOLOGY AND NEOPLASIA.* 2012 Epub ahead of print. PMID 22350980

Howe, E. N. Dawn R. Cochrane and **J.K. Richer**. Targets of miR-200c mediate suppression of cell motility and anoikis. BREAST CANCER RESEARCH. 2011 Apr 18;13(2):R45. PMID: 21501518. *Highlighted by DC Radisky BREAST CANCER RESEARCH Jun 10;13(3):110

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Cochrane DR, Cittelly DM, Howe EH., Spoelstra NS, McKinsey EL, LaPara K, Elias A, Yee D, and **JK Richer**. MicroRNAs Link Estrogen Receptor alpha Status and Dicer Levels in Breast Cancer. HORMONES AND CANCER. 2010. 1(6): 306-319. PMID:21761362

Cochrane DR**,** Howe EN, Spoelstra NS and **Jennifer K. Richer** Loss of miR-200c: A Marker of Aggressiveness and Chemoresistance in Female Reproductive Cancers. J. ONCOLOGY. 2009. Epub 2009 Dec 15.PMID: 20049172

Cochrane DR, Spoelstra NS, Howe EN, Nordeen SK and **JK Richer**. MicroRNA-200c Mitigates Invasiveness and Restores Sensitivity to Microtubule-Targeting Chemotherapeutic Agents. MOLECULAR CANCER THERAPEUTICS. Epub May 12 2009.PMID: 19435871

**Abstracts (Poster Presentations)**

Erin N. Howe and Jennifer K. Richer. miR-200c Targets a TrkB/NTF3 Autocrine Signaling Loop to Suppress Anoikis Resistance. AACR Special Conference on Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications. October 2011.

Erin N. Howe, Dawn R. Cochrane Diana Cittelly and Jennifer K. Richer, MicroRNA-200c Reverses EMT and Restores Sensitivity to Anoikis. Era of Hope Meeting, Orlando, FL. July, 2011.

Erin N. Howe, Dawn R. Cochrane and Jennifer K. Richer. miR-200c Directly Targets Multiple Non-epithelial Genes Involved in Motility and Anoikis Resistance. Rocky Mountain Reproductive Sciences Symposium.April, 2010.

Erin N. Howe, Dawn R. Cochrane, Nicole S. Spoelstra and Jennifer K. Richer. Insurance against EMT: MiR-200c directly targets multiple non-epithelial genes involved in motility and anoikis resistance. AACR Special Conference on EMT and cancer progression and treatment. February 2010.

Dawn R. Cochrane, Nicole S. Spoelstra, Erin N. Howe, Annie Jean, Steve K. Nordeen and Jennifer K. Richer. MicroRNA-200c Mitigates Invasiveness and Restores Chemosensitivity in Aggressive Endometrial, Ovarian and Breast Cancers. AACR Annual Meeting. April 2009.

Erin N. Howe, Dawn R. Cochrane and Jennifer K. Richer. Identification of Direct Targets of miR-200c other than ZEB1 and ZEB2. Poster #2648 AACR Annual Meeting, Denver, CO. April 2009.

Dawn R. Cochrane, Nicole S. Spoelstra, Erin N. Howe, Annie Jean, Steve K. Nordeen, and Jennifer K. Richer. MiR-200c Mitigates Invasiveness and Restores Chemosensitivity to Microtubule-Targeting Agents in Aggressive Endometrial, Ovarian and Breast Cancers. Keystone MiRNA and Cancer Meeting, June 2009.

Dawn R. Cochrane, Nicole S. Spoelstra, Erin N. Howe, Annie Jean, Steve K. Nordeen, and Jennifer K. Richer. MicroRNA-200c Mitigates Invasiveness and Restores Chemosenstivity in Endometrial Cancer. AACR-MRS Joint Conference on Metastasis. August 2008

**Honors and awards:**

Scholar-in-Training award recipient, AACR Special Conference on Advances in Breast Cancer Research:
Genetics, Biology, and Clinical Applications. October 2011.

Scholar-in-Training Award to Erin Howe, stipend to register and travel to AACR Special Conference on EMT and cancer progression and treatment. Arlington, VA, Feb 2010.

Scholar-in- Training Travel Award from the American Association for Cancer Research/National Cancer Institute for the joint AACR and Metastasis Research Society Meeting. Vancouver BC, August 2008.

**Invited Presentations**
**National:**

**Richer, JK,** EN Howe, DR Cochrane**,** and D Cittelly. Invited lecture "MicroRNAs that Regulate EMT in Breast and Gynecological Carcinomas"for Educational Section on EMT and Stem Cells in Cancer Progression. **Invited oral presentation for educational session. AACR 102nd Annual Meeting**. Orlando, FL. April, 2011

**Richer, JK.** Invited lecture "MicroRNAs that regulate EMT and tumor progression in breast and gynecological cancers" for the **MD Anderson Annual Uterine Cancer Biology Symposium** for the MD Anderson Gynecologic Cancer SPORE, May 19-20th, 2011.

**Richer, JK.** Invited lecture **"**miRNAs as guardians of the epithelial phenotype" **Gordon Conference on Mammary Gland Biology**. June, 2011

**Erin N. Howe**, Dawn R. Cochrane, Nicole S. Spoelstra and Jennifer K. Richer. **Invited Short Talk** "Insurance against EMT: MiR-200c directly targets multiple non-epithelial genes involved in motility and anoikis resistance. AACR Special Conference on EMT and cancer progression and treatment. Arlington Virginia, February 2010.

**Richer, JK,** EN Howe, and DR Cochrane. **Invited Short Talk.** MicroRNAs Differentially Expressed in Luminal versus Triple Negative Breast Cancer Control Estrogen Receptor alpha and Growth Factor Receptor Expression and Aspects of Tumor Metabolism. Keystone Symposia: Nuclear Receptors: Signaling, Gene Regulation and Cancer. March, 2010.

**Richer, JK,** EN Howe, and DR Cochrane. **Invited Symposia Oral Presentation** in Post-translational regulation of EMT Session. "Loss of microRNA-200c, a marker of EMT aggressiveness and chemoresistance in female reproductive cancers. The International Symposia on Epithelial to Mesenchymal Transition. Fourth Annual Meeting, Tucson, AZ Sept, 2009.

**Dawn R. Cochrane**, Nicole S. Spoelstra, Erin N. Howe, Annie Jean, Steve K. Nordeen, and Jennifer K. Richer. **Invited Short Talk** "MicroRNA-200c Mitigates Invasiveness and Restores Chemosenstivity in Endometrial Cancer." AACR-MRS Joint Conference on Metastasis. August 2008

**Local:**

**Richer, JK**. Cancer Biology Graduate Program Retreat – "MicroRNAs control distinguishing characteristics of breast cancer subtypes." 2010 Feb 9th

**Richer, JK** Cancer Center – Hormone Related Malignancies Retreat: Two miRNA Families Influence the Clinical Behavior of Breast and Gynecological Cancers 2011 March 25th

**Erin N. Howe** and Jennifer K. Richer. miR-200c Targets a TrkB/NTF3 Autocrine Signaling Loop to Suppress Anoikis Resistance in Breast Cancer. University of Colorado, Anschutz Medical Campus, Program in Cancer Biology annual update talk. January 2012.

**Erin N. Howe** and Jennifer K. Richer. miR-200c Targets TrkB and NTF3 to Suppress Anoikis Resistance in Breast Cancer. University of Colorado, Anschutz Medical Campus, Department of Pathology, Grand Rounds. June 2011.

**Patents**

2009 U.S. Provisional Application for United Stats Letters Patent *UTEC*:021USP1 "Micro RNAs Dysregulated in Triple-Negative Breast Cancer Inventors: Jennifer Richer, Dawn Cochrane, Steve Anderson

**Degrees:** Erin Howe, Cancer Biology Graduate Program will graduate with her doctorate in the summer 2012.

**Cell lines**

MDA-MB-231 with ZEB1 shRNA lentiviral vector or controls. MDA-231 cells were infected with three lentiviral constructs expressing shRNAs that putatively target ZEB1. As control, empty vector and shRNA targeting luciferase were used. Stable cells were selected using puromycin resistance. Only one of the shZEB1 constructs decreased ZEB1 levels significantly, shZEB1 #2. Due to the reciprocal repression between ZEB1 and miR-200c, these cells also express high levels of miR-200c.

Deliverables: MDA-231 and BT549 cells expressing pcQXIP, pcQXIP-mIkBa, shneg, shTrkB 195114, shTrkB 2242, shNTF3 58853, shNT3 58854

MCF7 and T47D cells expressing pCDNA3.1, pCDNA3.1-TrkB

**Animal models** –none yet

**Additional Funding Obtained based on this work:**

**NRSA** (mentor for Erin Howe, doctoral candidate, Cancer Biology Program, Richer lab)
NIH NCI
09/01/2011- 08/31/2013
**Title**: miR-200c Mediates Suppression of Anoikis Resistance by Targeting an Autocrine Signaling Pathway
**Goal:** to determine how miR-200c enhances anoikis sensitivity in breast cancer cells through targeting of a TrkB/NTF3 signaling pathway.

**CONCLUSION**
We have identified numerous direct and indirect targets of microRNA 200c and we have confirmed at least 5 as direct targets. These are all genes and their protein products that should not be expressed in normal epithelial cells, but that become expressed in high grade, very de-differentiated carcinomas that have undergone epithelial to mesenchymal transition (EMT). Some of these targets may, either individually or in combination, be responsible for the ability of miR-200c to dramatically reduce migration and invasion. We have demonstrated in our second paper that this ability is not due to restoration of E-cadherin because in some cells, miR-200c E-cadherin does not come back on following re-introduction of miR-200c (which may be either because E-cadherin is mutated or silenced by methylation); however, migration and invasion are still equally well reduced in these cases. We also, in our first paper, identified class III beta tubulin as the target responsible for the ability of miR-200c to reduce resistance to paclitaxel and other microtubule targeting agents. Lastly, we verify additional direct targets of miR-200c such as fibronectin and NTRK2 [3] and we believe that the latter is responsible for the ability of miR-200c to reverse anoikis resistance by reducing the protein TrkB and by also directly targeting NFF3, a ligand for TrkB (manuscript in preparation). We have thus contributed substantially to the important body of literature on the importance of miR-200c in affecting multiple steps in the metastatic cascade, and have summarized this nicely in a recent review[15]. We are also studying the effects of miR-200c on polarity and differentiation in 3D culture to determine whether we can use miR-200c to render tumors more well differentiated and less aggressive. These experiments as well as animal experiment to conclusively demonstrate the effects of this miRNA on metastasis are still underway.

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# CYTOGENETICS REPORT

**Cytogenetics Laboratory Numbers:** 09CY001-006
**Specimen IDs:** MIR200-T47D, MIR200-MCF7, MIR200-MDA231, MIR200-BT549, RP11-367L22, RP11-425E15
**Submitted by:** Dr. Jennifer Richer
**Received on:** January 7, 2009
**Sample type:** 4 breast cancer cell lines in culture and pure DNA from two BAC clones
**Test requested:** Evaluate status of genomic sequences carried by the BAC clones RP11-367L22 and RP11-425E15 in breast cancer cell lines

**PROCEDURE**

DNA in solution was received from two clones and due to low concentration DNAs were subjected to whole genome amplification using the Repli-G Kit (Qiagen, Valencia, CA) per manufacturer's instructions. Both BAC clones have particularly large human inserts: RP11-367L22 carries 194025 bp and RP11-425E15 carries 183090 bp. One ug of each DNA was labeled with SpectrumRed SR (RP11-367L22) and SpectrumGreen SG (RP11-425E15) conjugated dUTPs using the Vysis Nick Translation Kit (Abbott Molecular, Des Plaines, IL) according to the manufacturer's protocol. Labeled DNA was ethanol precipitated with herring sperm and the pellet was resuspended in 20ul of c-DenHyb (Insitus Biotechnologies, Albuquerque, NM). The newly labeled probes were tested on normal controls to verify mapping and quality of signal. RP11-367L22 mapped accurately to 12p13.31 (Figure 1); however, RP11-425E15 also mapped to the same location. A new verified RP11-425E15 DNA received from the Richer laboratory February 5, 2009 was amplified and labeled with SG dUTPs using the technique described above. Test of the labeled new RP11-425E15 probe on normal controls confirmed correct mapping and excellent quality of signal (Figure 2).

For each clone, a probe mixture was prepared to use for a 113mm$^2$ hybridization area as follows. RP11-367L22/CEP 12 included 50ng of RP11-367L22-SR, 1.0 ul of diluted CEP12-SG (1.0ul CEP12 + 10ul CEP buffer + 2ul sterile water) and 2.5ul of cDenHyb. RP11-425E15/CEP 1 included 100 ng of RP11-425E15-SR, 0.5ul of diluted CEP1-SR (1.0ul CEP1 + 10ul CEP buffer + 2ul sterile water) and 2.5ul of cDenHyb. Both CEP probes were obtained from Abbott Molecular. It is worth to highlight that the test probe was labeled in red in one set (RP11-367L22/CEP 12) and in green in the other set (RP11-425E15/CEP 1) because CEP 1 was only commercially available with the red label.

Cultures of the cell lines were received on January 7, 2009 and harvested following standard protocol after incubation with Colcemid (Invitrogen, Carlsbad, CA) for 3 hrs at 37$^o$C. Cells were detached with 0.05% Trypsin-EDTA, hypotonized with 0.075 M KCl at 37$^o$C for 15 min and fixed with fresh methanol:acetic acid 3:1. Fixed cell pellets were dropped onto pre-cleaned slides.

Each cell line was submitted to a dual-target FISH assay per our standard protocol. The slides were treated in 70% acetic acid for 15-20 sec, followed by incubations in 0.008% pepsin/0.01M HCL at 37$^o$C for 3-4 min, in 1% formaldehyde for 8 min and were then dehydrated in an ethanol series. The appropriate probe mixture was applied to the selected hybridization area, covered with a glass coverslip and sealed with rubber cement. DNA denaturation was performed for 5-8 min at 85°C and hybridization was allowed to occur at 37°C for 17-18 hours. Post-hybridization washes were performed with 2xSSC/0.3% NP-40 at 72$^o$C for 2 min, RT 2xSSC for 2 min and the slides were dehydrated in ethanol series. Finally, 14ul of DAPI anti-fade (0.3 ug/mL in Vectashield Mounting medium) was applied to the slides and the areas covered with a 24x50mm coverslip.

Analysis was performed on epifluorescence microscope using single interference filters sets for green (FITC), red (Texas red), blue (DAPI), dual (red/green), and triple (blue, red, green) band pass filters. For each interference filter, monochromatic images were acquired and merged using CytoVision (Applied Imaging Inc).

**RESULTS**

The quality of the preparation and the intensity of the fluorescence signals were excellent in both interphase nuclei and metaphase spreads. A total of 20 metaphases and 100 interphase nuclei were analyzed per specimen and results are summarized in Tables 1-4. Descriptive statistics of the interphase analysis for both clones in four cell lines are indicated in Table 1 and 2 including specimen ID, mean, standard deviation, the percentage of cells with ≤2, with 3 and with ≥4 copies of the gene and control, the ratio of the gene/control and figure indicator. The results of metaphase analysis for both clones in four cell lines are indicated in Tables 3 and 4 including specimen ID, ploidy, description of chromosomes harboring signals and figure indicator.

Metaphase and interphase results were compatible to each other for all 4 cell lines. The mean copy number per interphase nuclei for RP11-367L22 ranged from 2.01 to 3.79. The mean copy number per interphase nuclei for RP11-425E15 ranged from 2.04 to 5.32. For RP11-367L22, both MDS231 and BT549 had balanced ratio gene to control (1.00 and 0.99 respectively), while MCF7 and T47D had unbalanced loss, 0.75 and 0.26 respectively, compared with the CEP12 control probe. For RP11-425E15, only BT549 had balanced ratio gene to control (1.09) where as T47D, MCF7, and MDA231 had unbalanced loss, 0.69, 0.72 and 0.75 respectively, compared with the CEP1 control probe.

Table 1. Descriptive statistics of the interphase analysis in breast cell lines hybridized with RP11-367L22 (R = red signal) and CEP12 (G = green signal) probe set.

| Specimen ID MIR200 | RP11-367L22 | | | | | CEP 12 | | | | | RP11-367L22/ CEP12 | Figure |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | % cells with ≤2 copies | % cells with 3 copies | % cells with ≥4 copies | Mean | SD | % cells with ≤2 copies | % cells with 3 copies | % cells with ≥4 copies | | |
| T47D | 2.01 | 0.17 | 98.0 | 2.0 | 0.0 | 7.73 | 0.63 | 0.0 | 0.0 | 100.0 | 0.26 | 3B |
| MCF7 | 2.99 | 0.58 | 11.0 | 83.0 | 6.0 | 3.99 | 0.58 | 0.0 | 11.0 | 89.0 | 0.75 | 4B |
| MDA231 | 2.04 | 0.37 | 91.0 | 9.0 | 0.0 | 2.04 | 0.37 | 91.0 | 9.0 | 0.0 | 1.00 | 5B |
| BT549 | 3.79 | 0.66 | 3.0 | 19.0 | 78.0 | 3.82 | 0.66 | 2.0 | 20.0 | 78.0 | 0.99 | 6B |

Table 2. Descriptive statistics of the interphase analysis in breast cell lines hybridized with RP11-425E15 (G = green signal) and CEP1 (R = red signal) probe set.

| Specimen ID MIR200 | RP11-425E15 | | | | | CEP 1 | | | | | RP11-425E15/ CEP1 | Figure |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | % cells with ≤2 copies | % cells with 3 copies | % cells with ≥4 copies | Mean | SD | % cells with ≤2 copies | % cells with 3 copies | % cells with ≥4 copies | | |
| T47D | 4.00 | 0.45 | 1.0 | 5.0 | 94.0 | 5.76 | 0.53 | 0.0 | 0.0 | 100.0 | 0.69 | 3D |
| MCF7 | 2.04 | 0.20 | 96.0 | 4.0 | 0.0 | 2.83 | 0.38 | 17.0 | 83.0 | 0.0 | 0.72 | 4C |
| MDA231 | 2.20 | 0.45 | 82.0 | 16.0 | 2.0 | 2.92 | 0.56 | 14.0 | 84.0 | 2.0 | 0.75 | 5C |
| BT549 | 5.32 | 0.92 | 0.0 | 5.0 | 95.0 | 4.88 | 0.73 | 0.0 | 3.0 | 97.0 | 1.09 | 6C |

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Table 3. Results of metaphase analysis of breast cell lines hybridized with RP11-367L22 (R = red signal) and CEP12 (G = green signal) probe set.

| Specimen ID (FISH assay) | Ploidy | Description of chromosomes harboring signal | Figure |
|---|---|---|---|
| MIR200-T47D (09017.3) | Hyper 5n/Hypo 6n | 2 copies of apparently normal chromosome 12, 2 copies of metacentric add(12p) with only green, 4 copies of submetacentric del(12p) with only green; occasional (10%) submetacentric add(12q?) with only green | 3A |
| MIR200-MCF7 (09018.1) | Hyper 3n | 1 copy of apparently normal chromosome 12, 1 copy of der(12q+) with red and green, 1 copy of der(12q-) with red and green, and 1 copy of metacentric der(12) with only green | 4A |
| MIR200-MDA231 (09017.2) | Hypo 3n | 1 copy of apparently normal chromosome 12, 1 copy of der(12) or del(12q) with red and extra large green | 5A |
| MIR200-BT549 (09018.2) | 4n | 4 copies of apparently normal chromosome 12; 1 metaphase (5%) with 1 copy of submetacentric der(12) with only green | 6A |

Table 4. Results of metaphase analysis of breast cell lines hybridized with RP11-425E15 (G = green signal) and CEP1 (R = red signal) probe set.

| Specimen ID (FISH assay) | Ploidy | Description of chromosomes harboring signal | Figure |
|---|---|---|---|
| MIR200-T47D (09043.2) | Hyper 5n/Hypo 6n | 3-4 copies of apparently normal chromosome 1, 1-2 copies of submetacentric der(1) with only red | 3C |
| MIR200-MCF7 (09043.3) | Hyper 3n | 1 copy of apparently normal chromosome 1, 1 copy large der(1p-) with only red, 1 copy der(1p-) with only red, 1 copy of small submetacentric der(1) with only green; 1 metaphase (5%) with 1 copy of small submetacentric with only green | 4C |
| MIR200-MDA231 (09043.1) | Hypo 3n | 2 copies of apparently normal chromosome 1; in 50% metaphases 1 copy of metacentric der(1) with red and green; in 50% metaphases 1 copy of metacentric der(1) with del(1p) with only red; in 1 metaphase (5%) 1 submetacentric marker chromosome with only green | 5C |
| MIR200-BT549 (09043.4) | 4n | 4 copies of apparently normal chromosome 1, 1 copy of (1p) with 2 greens, 1-2 copies of submetacentric der(1q) with only red | 6D |

Table 5 summarizes the ploidy of each cell line and the "expected" gene copy number based on the ploidy and the mean copy number observed for each gene. Comparison of these numbers shows that for the genomic regions probed by the RP11-367L22 insert, two cell lines can be considered carrying these sequences in a balanced copy number - MCF7 and BT549 – and two cell lines display loss for the sequence– T47D and MDA231. For the genomic regions probed by RP11-425E15, one cell line - MDA231 - showed balanced copy number, two lines - T47D and MCF7 - exhibited low level of loss, and another line - BT549 - had genomic gain.

In addition, there was no suggestion of deletion or amplification of any of those regions based upon signal intensity. The intensity of each target signal within each cell line was uniform across normal and derivative chromosomes.

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Table 5. Summary of the ploidy of each cell line, the expected gene copy number based on the ploidy and the observed copy number for RP11-367L22 (12p13) and RP11-425E15 (1p36)

| **Cell Line** | **Ploidy** | **Expected copy number** | **RP11-367L22 12p13** | | **RP11-425E15 1p36** | |
|---|---|---|---|---|---|---|
| | | | **Mean copy** | **Status** | **Mean copy** | **Status** |
| M1R200-T47D | 5+/6- | 5-6 | 2.01 | loss | 4.00 | low level loss |
| M1R200-MCF7 | 3+ | 3-3.5 | 2.99 | balanced | 2.04 | low level loss |
| M1R200-MDA231 | 3- | 2.5-3 | 2.04 | low level loss | 2.20 | balanced |
| M1R200-BT549 | 4 | 4 | 3.79 | balanced | 5.32 | gain |

**Marileila Varella Garcia, Ph.D.**
**February 24, 2009**

**List of personnel receiving pay from research effort:**

Jennifer Richer, Ph.D. Associate Professor

Steve Nordeen, Ph.D. Professor (first year only)

Cochrane D, Ph.D. Post-doctoral fellow

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miR-200c targets a TrkB/NTF3 autocrine signaling loop to suppress anoikis resistance

Erin N. Howe, Jennifer K. Richer.

**Background**: Loss of miR-200c has been linked to epithelial to mesenchymal transition (EMT) and progression of several types of cancer. By targeting several mesenchymal genes, miR-200c represses EMT associated phenotypes, including migration, invasion and stemness. We have demonstrated that miR-200c suppresses anoikis resistance in breast and endometrial cancer cells. Anoikis is a form of apoptosis induced when cells are detached from their native extracellular matrix (ECM). Resistance to anoikis is necessary for carcinoma cells as they travel through the vasculature or lymphatics during metastasis. We have shown that miR-200c suppresses anoikis resistance by targeting the neurotrophic tyrosine kinase receptor, TrkB. The Trk family of receptor tyrosine kinases (including TrkA, TrkB, TrkC and the orphan receptor p75) has a well-established role in neuronal differentiation and survival. TrkB has been linked to anoikis resistance in multiple types of cancer, including ovarian, breast and head and neck; however, all studies to date have used the canonical ligand, brain derived neurotrophic factor (BDNF). Another neurotrophic factor, neurotrophin 3 (NTF3), is also capable of binding TrkB and we noted that the 3' UTR of NTF3 contains two putative miR-200c binding sites, indicating that it may be repressed by miR-200c. This led us to the hypothesis that miR-200c directly represses both the receptor and ligand in an aberrant autocrine signaling loop to suppress anoikis resistance in breast cancer.

**Results**: We began by investigating the necessity of a ligand for TrkB to induce anoikis resistance in a breast cancer model. Anoikis sensitive MCF7 and T47D breast cancer cells were stably selected for expression of TrkB, and given increasing doses of BDNF or NTF3 prior to being plated in suspension. We found that TrkB does indeed require a ligand for these cells lines to resist anoikis, and NTF3 was able to induce the same degree of anoikis resistance as BDNF. This indicates that, although BDNF is the preferred ligand in a neuronal setting, NTF3 is also able to induce TrkB mediated anoikis resistance in breast cancer cells. To determine the relative importance of TrkB and NTF3 in miR-200c mediated suppression of anoikis resistance, we utilized anoikis resistant MDA-231 and BT549 breast cancer cells stably selected for expression of shRNAs targeting TrkB or NTF3. Cells were plated in suspension and we show that cells with knockdown of either the receptor or the ligand are less anoikis resistant. Further, cells with robust knockdown of either TrkB or NTF3 did not exhibit further repression of anoikis resistance following restoration of miR-200c. Taken together, this data indicates that miR-200c suppresses anoikis resistance by repressing the TrkB/NTF3 signaling axis in breast cancer cells. Importantly, we utilize a dual luciferase assay to demonstrate that miR-200c directly targets NTF3 via two binding sites in the 3' UTR. Although a previous study failed to detect expression of TrkB or NTF3 in a panel of breast cancer cell lines, we show that TrkB and NTF3 are up-regulated in suspended MDA-231 and BT549 cells. Interestingly, the anoikis sensitive MCF7 and T47D cells failed to up-regulate either TrkB or NTF3, indicating that though TrkB and NTF3 are not required for adherent growth, they may be necessary for survival in suspension.

**Conclusions:** In aggressive breast cancer cell lines, miR-200c potently suppresses anoikis resistance by directly targeting both TrkB and NTF3. Furthermore, anoikis resistant cells actively up-regulate TrkB and NTF3 to survive in suspension, an effect blocked by miR-200c. Our data suggests that through targeting of this autocrine loop, miR-200c might restore anoikis sensitivity and suppress breast cancer metastasis more powerfully than targeting either component alone.

Rocky Mountain Reproductive Sciences Symposium.April 2010.

**MiR-200c directly targets multiple non-epithelial genes involved in motility and anoikis resistance.** *Erin N. Howe, Dawn R. Cochrane and Jennifer K. Richer*

Breast and uterine cancer are the most common reproductive cancers for women. The more aggressive triple negative breast cancers and type 2 endometrial cancers do not express their hormone receptors (estrogen receptor, progesterone receptor and Her2) so there are currently no targeted therapeutics available to women with these diseases. Additionally, these aggressive cancers are believed to have undergone an epithelial to mesenchymal-like transition (EMT), rendering them more motile, less sensitive to anoikis (detachment induced apoptosis), and less sensitive to chemotherapeutics. Background: MiR-200c directly targets ZEB1, a transcription factor known to repress E-cadherin and other key determinates of epithelial identity and polarity. Restoration of miR-200c to high grade carcinoma cell lines results in complete repression of ZEB1/2, restoration of E-cadherin protein expression, dramatic reduction in migration and invasion, and increased sensitivity to microtubule targeting chemotherapeutics (Cochrane *et al* 2009). We hypothesized that repression of ZEB1 alone could not account for all of these effects and that miR-200c must target other genes that contribute to its myriad of effects on cancer cell biology. We now demonstrate that miR-200c directly targets many "non-epithelial" genes typically only expressed in cells of mesenchymal or neuronal origin. Results: We performed microarray analysis on Hec50 endometrial cancer cells (an aggressive type 2 endometrial cancer cell line that has lost miR-200c and undergone EMT) either mock transfected or transfected with miR-200c mimic or scrambled control mimic. We identified genes statistically significantly altered by at least two fold following restoration of miR-200c, 18 of which have putative target sites for miR-200c binding. These genes, including fibronectin 1 (FN1), class III beta-tubulin (TUBB3), neurotrophic tyrosine receptor kinase type 2 (NTRK2), leptin receptor (LEPR) and Rho GTPase activating protein 19 (ARHGAP19), are all typically expressed only in non-epithelial cells. We have validated each of these genes as direct targets of miR-200c. Finally, we observe that the endogenous mRNA encoding these genes is decreased upon restoration of miR-200c. FN1, NTRK2, LEPR and ARHGAP19 are involved in cell movement and may contribute to the decreased migration and invasion observed upon restoration of miR-200c even when E-cadherin is not re-expressed. NTRK2 is a potent suppressor of anoikis in breast and ovarian cancers and indeed we demonstrate that restoration of miR-200c enhances anoikis, leading to over a 1.5 fold increase in cell death in multiple cell lines. TUBB3 is abnormally expressed in tumors resistant to taxanes and we show that direct repression of TUBB3 is the mechanism whereby miR-200c increases sensitivity to microtubule targeting chemotherapeutics. We also found that the gene encoding the actin binding protein moesin (MSN1), a bioinformatically predicted target of miR-200c, is significantly decreased in the presence of miR-200c. Conclusions: Our data demonstrate that the function of miR-200c as a "guardian of the epithelial phenotype" and suppressor of EMT stems from its ability to repress numerous "non-epithelial" genes, resulting in a more well-differentiated epithelial state and decreased EMT-like phenotypes including invasive potential and anoikis resistance. Thus miR-200c is poised to be developed as a differentiation therapy for patients with aggressive breast and endometrial cancers.

Era of Hope Meeting, Orlando, FL. July, 2011.

**MicroRNA-200c Reverses EMT and Restores Sensitivity to Anoikis**

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**Background:** MicroRNA-200c directly targets and represses ZEB1 and ZEB2. These transcription factors cause epithelial to mesenchymal transition (EMT) by repressing E-cadherin and other key determinates of epithelial identity and polarity. Restoration of miR-200c to breast cancer cells that have undergone EMT results in repression of ZEB1, re-expression of E-cadherin, dramatic reduction in migration and invasion, and increased sensitivity to microtubule targeting chemotherapeutics. We hypothesized that miR-200c maintains the well-differentiated epithelial phenotype by directly inhibiting translation of additional targets other than ZEB1/2 that play a role in multiple steps in the metastatic cascade including loss of polarity, migration/invasion, drug resistance, and resistance to anoikis.

**Results:** We identified 18 genes significantly repressed and predicted to be direct targets of miR-200c. These genes, including fibronectin 1 (FN1), class III beta-tubulin (TUBB3), neurotrophic tyrosine receptor kinase type 2 (NTRK2 or TrkB), leptin receptor (LEPR), moesin (MSN1) and Rho GTPase activating protein 19 (ARHGAP19), are all typically expressed only in non-epithelial cells, such as fibroblasts or neurons, but become expressed in highly de-differentiated breast cancers and other carcinomas that have undergone EMT. Utilizing luciferase reporter assays and mutational analysis, we validated each of these genes as direct targets of miR-200c and miR-200c leads to a dramatic decrease in the endogenous mRNA and protein levels of the targets. We demonstrate that restoration of miR-200c restores anoikis sensitivity to suspended cells. TrkB is a potent suppressor of anoikis (detachment induced apoptosis) in breast and ovarian cancer models. Addition of TrkB that cannot be targeted by miR-200c reversed the ability of miR-200c to suppress anoikis. Interestingly, neurotrophin 3 (NTF3), a ligand for TrkB, contains 2 miR-200c binding sites and is down regulated upon restoration of miR-200c as well. Thus, loss of miR-200c in carcinoma cells allows inappropriate expression of both TrkB and NTF3, setting up an autocrine loop that results in anoikis resistance, which can be reversed by addition of miR-200c.

**Conclusions:** Our data demonstrate that miR-200c directly targets and repressing a program of genes not typically expressed in well-differentiated epithelial cells. We identify a novel function of miR-200c, the ability to suppress anoikis resistance, an important yet understudied step in the metastatic cascade.

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# The miR-200 and miR-221/222 microRNA Families: Opposing Effects on Epithelial Identity

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**Abstract** Carcinogenesis is a complex process during which cells undergo genetic and epigenetic alterations. These changes can lead tumor cells to acquire characteristics that enable movement from the primary site of origin when conditions become unfavorable. Such characteristics include gain of front-rear polarity, increased migration/invasion, and resistance to anoikis, which facilitate tumor survival during metastasis. An epithelial to mesenchymal transition (EMT) constitutes one way that cancer cells can gain traits that promote tumor progression and metastasis. Two microRNA (miRNA) families, the miR-200 and miR-221 families, play crucial opposing roles that affect the differentiation state of breast cancers. These two families are differentially expressed between the luminal A subtype of breast cancer as compared to the less well-differentiated triple negative breast cancers (TNBCs) that exhibit markers indicative of an EMT. The miR-200 family promotes a well-differentiated epithelial phenotype, while high miR-221/222 results in a poorly differentiated, mesenchymal-like phenotype. This review focuses on the mechanisms (specific proven targets) by which these two miRNA families exert opposing effects on cellular plasticity during breast tumorigenesis and metastasis.

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**Keywords** miR-200 · miR-221 · miR-222 · EMT · MET · Breast cancer

**Abbreviations**

| | |
|---|---|
| EMT | Epithelial to mesenchymal transition |
| ZEB1/2 | Zinc finger E-box binding homeobox 1/2 |
| UTR | Untranslated Region |
| MET | Mesenchymal to epithelial transition |
| MDCK | Madin-Darby Canine Kidney |
| iPSC | Induced pluripotent stem cell |
| TGF-β | Transforming growth factor beta |
| PDGF | Platelet derived growth factor |
| EGFR | Epidermal growth factor receptor |
| NCI | National Cancer Institute |
| VEGF | Vascular endothelial growth factor |
| ER | Estrogen receptor |
| MMTV | Murine mammary tumor virus |
| TRPS1 | trichorhinophalangeal 1 |
| PLZF | promyelocytic leukemia zinc finger |

## Introduction

miRNAs are small (18–25 nucleotide) non-coding RNAs that regulate gene expression post-transcriptionally by binding to the 3′ untranslated region (UTR) of target messenger RNAs (mRNAs) [1], and inhibiting translation or targeting the mRNA for degradation [2]. The extent to which miRNAs regulate the human transcriptome is still under investigation; however, miRNAs can target hundreds of genes, suggesting that their regulatory role may be as significant as that of transcription factors. miRNAs are differentially regulated during development [3–5]. Controlled epithelial to mesenchymal transition (EMT) is a normal process in



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development, required for processes such as gastrulation, mammary gland branching, and neural crest formation (reviewed in [6]). However, EMT is a pathological event in cancer that contributes to the gain of aggressive characteristics that facilitate metastasis [7–10]. In cancer EMT, carcinoma cells do not become mesenchymal cells, although there can be a marked loss of epithelial hallmarks and a shift toward mesenchymal and even neuronal gene expression. It is widely believed that acquisition of these characteristics can allow tumor cells to become motile, invasive, and able to intravasate into the blood and lymph vessels and survive the metastatic journey. Transcription factors, such as Twist, Snai1, and ZEB1/2 (Reviewed in [11]) regulate both normal and oncogenic EMT. ZEB1 (zinc finger E-box binding homeobox 1) and ZEB2 (also known as SIP1) directly repress the adherens junction protein E-cadherin [12–14] and other genes involved in polarity and epithelial identity [15, 16].

ZEB1/2 are post-transcriptionally controlled by the miR-200 family of miRNAs [17–19], and ZEB2 is indirectly controlled by the miR-221 family [20]. Indeed, recent studies have identified the miR-200 and miR-221 families as differentially expressed in carcinomas, particularly in breast cancer [20, 21]. Specifically, the miR-200 family is high in the luminal breast cancer subtypes, while miR-221/222 is overexpressed in triple negative breast cancers (TNBCs), particularly those that have undergone EMT. These miRNAs control expression of many genes that define the EMT-like phenotype and likely affect tumor behavior and clinical outcome by influencing metastatic potential. Thus, in this review we focus on the opposing roles of these two miRNA families in controlling differentiation state or epithelial identity in breast cancer.

## miR-200 Protection of the Epithelial Phenotype

### miR-200 Family Regulation of EMT in Breast Cancer

The miR-200 family of miRNAs is comprised of two polycistronic clusters—miR-200c and miR-141 on chromosome 12 and miR-200b, miR-200a and miR-429 on chromosome 1. miR-200a and miR-141 share a seed sequence, while miR-200b, miR-200c and miR-429 also share a seed sequence, which differs from that of miR-200a/141 by one nucleotide. Because of their sequence similarity, the miRNAs are predicted to share gene targets; however, there is evidence that the two clusters control different regulatory networks even in the same model. In MDA-MB-231 cells the miR-200bc/429 cluster induces G2/M arrest, while miR-200a/141 induces G0/1 arrest [22]. Additionally, miR-200c directly targets and down-regulates the transcription factor ZEB1, while miR-200a does not [23].

The miR-200 family was first discovered to directly target and down-regulate the E-cadherin transcriptional repressors ZEB1 and ZEB2, leading to restoration of an epithelial phenotype in breast cancer cell lines, characterized by an increase in E-cadherin expression, and decreased migration and invasion [17–19]. Expression of the miR-200 family correlates with an epithelial-like phenotype in the National Cancer Institute (NCI) panel of 60 cancer cells lines [19], and suppresses EMT in several additional cancer models, including bladder [24], colorectal [25, 26], and lung [27–30]. Although genes encoding ZEB1/2 are the best-studied targets of the miR-200 family, the small consensus binding sequence of miRNAs results in many bioinformatically predicted targets. The miR-200 family has now been confirmed to directly target other genes involved in various aspects of EMT. One aspect of EMT that has been particularly well studied is the increase in migratory and invasive capacity. Targeting and repression of the genes encoding ZEB1/2 by miR-200c and the resultant increase in E-cadherin decreases migration and invasion; however, direct targeting of genes encoding the actin cytoskeleton associated proteins WAVE3 [31] and MSN [32], and the extracellular matrix component FN1 [32] also contribute to suppression of motility and invasion. The miR-200 family also targets two genes involved in cell cycle control, RND3 [33] and FOG2 [34].

The power of miRNAs lies in their ability to target multiple genes that contribute to a pathway or phenotype. For instance, normal well-differentiated mammary epithelial cells exhibit hallmarks such as E-cadherin and hormone receptor expression, while poorly differentiated breast carcinoma cells loose these characteristics. When carcinoma cells revert towards a less-differentiated state, in addition to losing expression of epithelial hallmarks, they also inappropriately gain expression of proteins that confer the ability to move away from the primary tumor when conditions are harsh (hypoxia, lack of nutrients, and build-up of waste products). The tumor cells must also be able to resist anoikis in order to survive detachment from the basement membrane.

Anoikis resistance is a relatively poorly understood and understudied aspect of EMT. Anoikis is apoptosis induced when cells lose attachment to their native extracellular matrix (ECM), and resistance to anoikis is required for cancer cells to survive as they move away from the primary tumor, and travel through the vasculature or lymphatics to metastatic sites. Data from our lab demonstrate that miR-200c suppresses anoikis resistance through direct targeting of *NTRK2,* the gene encoding TrkB [32], a receptor tyrosine kinase involved in neuronal development and differentiation. TrkB was first associated with anoikis resistance when it was isolated from a cDNA library screen designed to identify genes capable of conferring anoikis resistance to normal intestinal epithelial cells [35]. TrkB is involved in anoikis resistance in breast cancer [32, 35–38] and is specifically expressed in TNBCs that have undergone EMT, but not luminal A lines [32].

Resistance to chemotherapy is a critical aspect of tumorigenesis also associated with acquisition of an EMT phenotype. The miR-200 family has been found to be involved in maintaining sensitivity to two classes of chemotherapeutics to date, microtubule targeting agents, and DNA damaging drugs. In aggressive cancer cells resistant to taxanes, restoration of miR-200c increases sensitivity due to its direct targeting of *TUBB3,* the gene encoding class III beta tubulin [39, 40]. TUBB3 is a tubulin isoform aberrantly expressed in several types of carcinomas [41–43], including breast [44, 45], that leads to resistance to taxanes (Reviewed in [46]). Additionally, the miR-200 family is down-regulated in MCF7 cells selected for resistance to cisplatin [47], or doxorubicin [48]. Indeed, miR-200 expression correlates with sensitivity to EGFR blocking agents in bladder cancer, and restoration of miR-200 family members increased sensitivity to EGFR inhibitors in mesenchymal-like cell lines [49]. Additionally, lower expression of miR-200c was observed in a panel of 39 breast cancer patients resistant to chemotherapy [48]. The authors speculate that these effects may be due to the predicted targeting of the multidrug resistance gene 1 by miR-200c, but this remains to be proven. Finally, miR-200c directly targets FAP-1, leading to restoration of sensitivity to CD-95 (Fas)—mediated apoptosis [50]. Thus, the miR-200 family exerts multi-level control over apoptosis in epithelial cells. The family promotes sensitivity to natural apoptotic stimuli, including loss of adhesion and Fas signaling, while also preventing resistance to several classes of therapeutic agents.

While not classically thought of as a characteristic of EMT, an overall decrease in miRNA abundance is found in aggressive cancer cells [51, 52]. Dicer, an enzyme involved in the maturation of miRNAs, is often low in cancers that have undergone EMT [53]. While the mechanism remains to be elucidated, we demonstrated that restoration of miR-200c to TNBC cell lines causes an increase in Dicer protein [21]. Since relatively high levels of Dicer and overall miRNA abundance are characteristic of normal epithelial cells, this is a unique mechanism through which the miR-200 family promotes an epithelial phenotype.

In addition to regulation of EMT, there is emerging evidence that the miR-200 family plays a role in epigenetic regulation and inhibition of stem cell-like qualities in breast, prostate [54, 55], and colorectal cancer cells [26]. Expression of both miR-200 family clusters is down-regulated in stem cells isolated from normal human breast, and murine mammary glands, as well as in stem cells isolated from breast cancer patients [56]. Inhibition of miR-200 leads to an enrichment of the stem cell population, and up-regulation of the miR-200b direct target Suz12, a subunit of the polycomb repressor complex. Increased Suz12 leads to trimethylation and polycomb-mediated repression of the E-cadherin promoter [57]. Another direct target, the gene encoding class III histone deacetylase, SIRT1, deacetylates histone H3 at the E-cadherin promoter, and miR-200 mediated repression of SIRT further relieves repression of E-cadherin [58]. The miR-200 family also directly targets and represses *Bmi1*, allowing further repression of stemness [26]. Additionally, expression of miR-200c inhibits clonal expansion of stem cells, and prevents tumor formation from patient-derived breast cancer stem cells transplanted into mice [56]. Finally, two important stem cell factors, Sox2 and KLF4 have been found to be down-regulated following restoration of miR-200c [26]. Thus, the miR-200 family controls multiple genes that repress cancer stem cells, leading to restoration of an epithelial phenotype and decreased aggressiveness. The genes and aggressive phenotypes repressed by the miR-200 family are detailed in Fig. 1.

The miR-200 family is highly expressed in luminal A breast cancer cell lines and lost in TNBC lines [21]; however, data from primary and metastatic breast cancer samples are not as clear. Based on the cell line data, it was expected that the miR-200 family would be down-regulated in aggressive tumors and metastases. While this is true in some models, and restoration of miR-200 to a TNBC cell line prevents metastases [59], in other models the miR-200 family positively correlates with metastases [60, 61]. Consistent with the theory that miR-200c positively correlates with a well-differentiated phenotype, the miR-200 family is very low in the poorly differentiated claudin-low subtype of breast cancer, while expression of ZEB1/2, vimentin, and Twist are high and these tumors are enriched for tumor initiating cells, suggesting that the miR-200 family must be down-regulated for formation of an aggressive subpopulation of tumor cells [62]. However, while several profiling studies found that expression of the miR-200 family is lost between normal breast tissue and malignant breast cancers [18, 63] one profiling experiment [64], comparing luminal A, luminal B, basal-like and malignant myoepithelioma, revealed that while the miR-200 family is highly expressed in luminal tumors, it is also highly expressed in basal-like tumors. Only malignant myoepitheliomas showed down-regulation of the miR-200 family, which is consistent with a strong EMT phenotype [64].

Expression of the miR-200 family in metastatic disease has been even more contested. While one group found the miR-200 family to be down-regulated between matched primary versus metastatic breast, colon, lung and bladder cancers [65], another showed that the miR-200 family is over-expressed in matched metastases, and that higher than median expression of several family members correlates with decreased progression free survival in estrogen receptor (ER) positive breast tumors [61]. In contrast, high expression of miR-200b, and low expression of Suz12 can

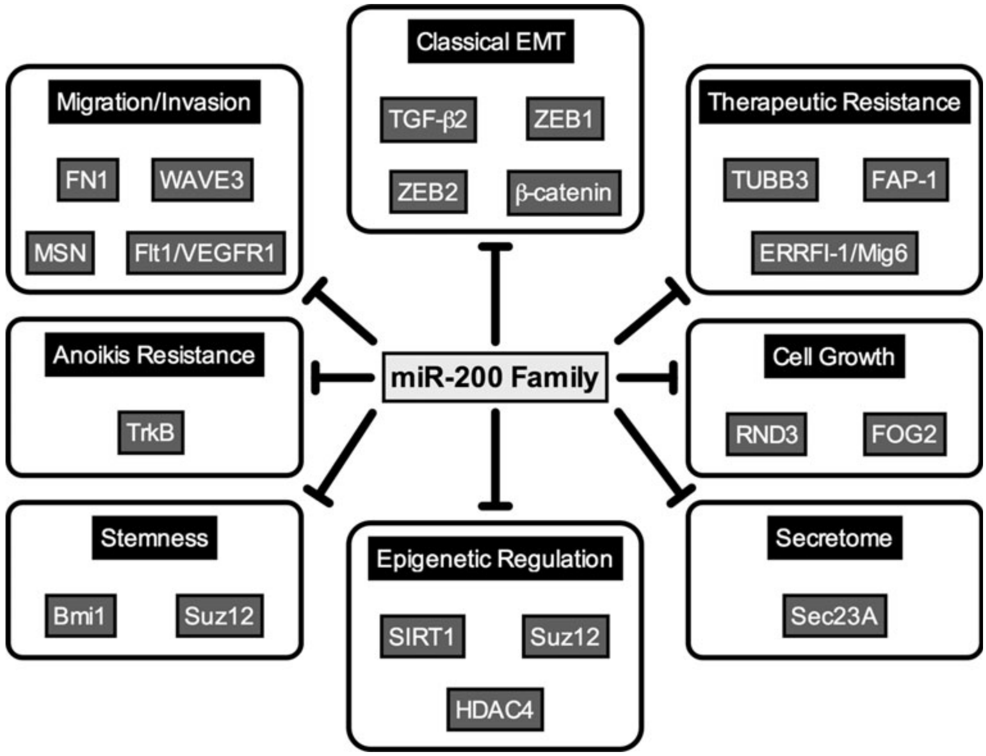


**Fig. 1** Direct targets of the miR-200 family. Members of the miR-200 family directly target and down-regulate genes involved in a variety of processes that contribute to tumorigenesis and metastasis. References are included in the text

distinguish primary breast tumors from metastases, which express low miR-200b and high Suz12 [57]. Further complicating the matter are two studies performed in syngeneic mouse mammary carcinoma models. In one study, using the 4T1 panel of cell lines, expression of miR-200 in a non-metastatic cell line increased metastasis [60]. Forced expression of miR-200c and miR-141, or all members of the miR-200 family led to increased metastasis in a similar model, the 4TO7 cell line [61]. These studies suggest that expression of the miR-200 family may induce mesenchymal to epithelial transition (MET) during the metastatic cascade. Induction of MET may be necessary for colonization of cells at the metastatic site, which would be consistent with increased expression of the miR-200 family. It is also possible that EMT is not required for metastasis in these models. Another possible explanation is that there are differences in the rate limiting steps of the metastatic cascade across models, which could affect the necessity of MET in colonization. Finally, regulated expression of miR-200 may be important for phenotypic plasticity, and may allow cells to transition between epithelial and mesenchymal states as needed.

## miR-200 Family in Plasticity

There is mounting evidence that both EMT and MET are important in the progression of carcinomas, and that carcinoma cells exhibit increased plasticity, allowing them to transition as necessary. Both EMT and MET are required for proper development, and the role of the miR-200 family in transitions between the epithelial and mesenchymal states is becoming clear. During embryonic stem cell differentiation, the miR-200 family is down-regulated by Snai1 and Wnt signaling, and forced expression of miR-200 leads to cells stalling at the epiblast-like stem cell stage of differentiation [66]. The miR-200 family is also regulated by c-Myc in differentiating embryonic stem cells [67].

Forced expression of miR-200c in epithelial cells of the developing mammary gland suppresses ductal growth [56], suggesting that plasticity is required for proper formation of the ducts. Similarly, forced expression of miR-200 in plastic, metastatic lung adenocarcinoma cells reversed plasticity, preventing the cells from undergoing EMT or metastasizing [68]. Manipulation of ZEB1/2 and the miR-200 family in Madin-Darby canine kidney (MDCK) cells leads to EMT and MET, respectively, but the states remain plastic and can be reversed [69]. miRNA profiling of embryonic stem cells, induced pluripotent stem (iPSC) cells, differentiated cells and cancer cells revealed that the pluripotent stem cells formed two clusters, irrespective of the origin of the cells (embryonic versus induced). The miRNAs that distinguished these groups also differentiated normal cells from cancer cells. Expression of miR-92 or miR-200 family members in iPSCs changed their classification status, leading the authors to suggest that the subdivision in pluripotent stem cell states does not reflect their origin, but rather miRNA and gene expression network [70]. Similarly, the miR-200 family is regulated during reprogramming of somatic cells into iPSCs [71]. Thus, the miR-200 family, as well as EMT-inducing transcription factors, must be expressed in the proper order to allow differentiation of embryonic stem cells.

### Regulation of the miR-200 Family

The most potent regulators of the miR-200 family are ZEB1 and ZEB2, which have been demonstrated to target E-boxes in the miR-200 cluster promoters [72, 73]. Another well recognized EMT inducer, transforming growth factor beta (TGF-β), has also been shown to reduce expression of the miR-200 family in transformed human breast epithelial cells [74], murine mammary epithelial cells [75], prostate cancer cells [76], and canine renal MDCK cells, a model of the epithelial phenotype [18, 77]. Indeed, treatment with TGF-β leads to hypermethylation of the miR-200 promoters, potentially through miR-200a-mediated direct targeting of the histone deacetylase SIRT1 [74]. Further study of the role of epigenetic regulation of the family revealed that the promoters are unmethylated in epithelial cells, and in cancer cells that express the family, but heavily methylated in fibroblasts and tumors that do not express the miR-200 family [78, 79]. Furthermore, the permissive epigenetic mark, histone H3 acetylation, is decreased at the miR-200 promoter in cancer cells lacking expression of the family [80], an epigenetic mark potentially influenced by miR-200a direct targeting of HDAC4. Together, this data indicates that while classical EMT-inducers control expression of the miR-200 family in tumorigenesis, epigenetic control is also important, and potentially forms feedback loops through miR-200 control of epigenetic regulators, including SIRT1, HDAC4, and Suz12.

Several other EMT inducers down-regulate the miR-200 family, including platelet derived growth factor (PDGF) [81], long-term treatment with the epidermal growth factor receptor (EGFR) inhibitor gemcitabine [82], and carcinogen induced tumorigenesis [83]. Interestingly, treatment of pancreatic cancer cells with curcumin, or the analog CDF, along with gemcitabine lead to increased miR-200 family expression [81, 84]. Additionally, Akt isoforms leads to differential miRNA expression profiles. Expression of only Akt2 dramatically decreases expression of the miR-200 family, while knockdown of Akt1 induced EMT by reducing expression of the miR-200 family. The authors suggest that the expression of miR-200 family members depends on the ratio of Akt1/Akt2, rather than the overall activity of Akt [85]. To date, the only known activators of miR-200 expression are the tumor suppressors p53 [86, 87], p63, and p73 [88], and ERα [89]. However, there are likely other positive-regulators of the miR-200 family.

## miR-221/222 Suppression of the Epithelial Phenotype

### miR-221/222 Expression in Breast Cancer and Other Carcinomas

miR-221 and miR-222 are found on the X chromosome and are expressed from a single transcript. For many cancer types, miR-221/222 are considered oncomiRs, and are overexpressed in tumor compared to normal tissue of origin. This expression pattern holds true in breast [63], prostate [90], gastric [91], bladder [92], papillary thyroid carcinoma [93], colorectal cancer [94], melanoma [95], and acute myeloid leukemia [96]. High miR-221/222 expression is associated with increased tumor grade [97, 98] and poor prognosis [99]. High miR-221 is found in prostate cancer cell lines, where it is associated with aggressive phenotypes, such as androgen-independence and neuroendocrine differentiation [90].

Several studies have demonstrated that miR-221/222 directly target ERα [21, 100, 101]. In breast cancer, miR-221/222 negatively correlate with ER status, and are more highly expressed in triple negative cell lines as compared to luminal [20, 21, 100] and the same holds true in clinical samples [21, 102]. Additionally, in the murine mammary tumor virus (MMTV)-c-myc mouse model of mammary carcinoma, miR-222 is increased during tumorigenesis [103]. However, some controversy exists, since one study observed that although miR-221 is overexpressed in TNBCs and is associated with poor disease-free and overall survival, there was no difference in miR-222 expression between breast cancer and normal epithelial tissue [99]. Additionally, another study found that miR-221 expression positively correlated with ER status in breast cancer patient samples, while miR-222 expression did not change between ER positive and ER negative samples [104]. Thus, as with the miR-200 family, although expression of miR-221/222 correlates strongly with specific phenotypes in vitro in breast cancer cell lines, more work is required to fully elucidate the role of the family in human tumors.

### miR-221/222 in EMT and Metastasis

Since miR-221/222 are often overexpressed in poorly differentiated, aggressive cancers, it stands to reason that these miRNAs play an active role in promoting EMT. Increasing miR-221 or 222 can affect various characteristics associated with EMT, including increased invasive capacity [90, 105], and anoikis resistance [106]. Low Dicer is characteristic of poorly differentiated cells and cells that have undergone EMT. In TNBC lines, miR-221/222 directly target and repress Dicer1 [21], leading to the possibility that aberrant expression of miR-221/222 leads to decreased Dicer, which in turn leads to a decrease in overall miRNA abundance.

Long term mammosphere culture of MCF7 cells induces EMT, with the resulting cells displaying a basal B phenotype [107]. The cells also exhibit increased expression of stem cell markers (CD44+/CD24-/low), and exhibited stem cell-like characteristics, including chemoresistance. qRT-PCR miRNA profiling demonstrates that miR-200c, -203 and -205 are decreased, while miR-221/222 are increased in the mammosphere cultured cells, with miR-222 increased 20-fold [107]. Thus, although further more exhaustive and rigorous genetic analysis of necessity and sufficiency remains to be performed, it appears that induction of EMT in luminal breast cancer cells involves decreased expression of the miR-200 family and increased expression of miR-221/222. Although miR-221/222 are high in both basal A and B breast cancer, their expression is higher in the basal B subtype, which has a more mesenchymal phenotype [20], consistent with the role of miR-221/222 in EMT. Forced expression of miR-221/222 in luminal breast cancer cells causes a decrease in E-cadherin and an increase in the mesenchymal marker vimentin [20]. Luminal cells expressing miR-221/222 gained a more mesenchymal morphology and had increased migratory and invasive capacity. Conversely, inhibition of miR-221/222 in basal-like cells promoted MET [108]. miR-221/222 promote a mesenchymal phenotype in part by directly targeting trichorhinophalangeal 1 (TRPS1), and keeping its levels low [20]. TRPS1 is a transcriptional repressor that binds to GATA sites that can promote MET [20], and is underexpressed in breast cancers with poor clinical outcome [109]. TRPS1 represses the mesenchymal transcription factor ZEB2 through a GATA site in its promoter. As ZEB2 is a repressor of E-cadherin, this provides a functional link between expression of miR-221/222 and repression of E-cadherin in basal breast cancers [20, 110].

## miR-221/222 Control of Proliferation

miR-221/222 positively influence cellular proliferation in many types of cancers. While there are several mechanisms through which increased growth rate is achieved, the best studied is direct targeting of $p27^{KIP1}$ [98, 111], and $p57^{KIP2}$ [112, 113]. In patient samples, miR-221 or miR-222 levels are often inversely correlated with $p27^{KIP1}$ [111, 114–116] or $p57^{KIP2}$ [94, 112]. Increasing the expression of miR-221 or miR-222 causes increased proliferation in vitro [111, 114], and increased tumor growth in xenograft tumor models [117]. Conversely, antagonizing miR-221/222 results in decreased proliferation both in vitro [94] and in vivo [118]. In one study, decreased tumor growth was achieved through in vivo administration of cholesterol modified anti-miR-221, which suggests that miR-221 can be a viable therapeutic target for the treatment of aggressive cancers [119].

Direct targets other than $p27^{KIP1}$ and $p57^{KIP2}$ can also mediate the proliferative effects of miR-221/222. In gastric cancer cells, the proliferative effects of miR-221/222 are partially due to their ability to directly target PTEN [105], and targeting of PTEN is also likely to play an important role in breast carcinomas. Additionally, miR-221/222 directly target ARH1 [120], a tumor suppressor protein decreased in many types of cancers [121–123]. Loss of ARH1 results in increased proliferation, colony formation and invasion [120]. Thus, miR-221/222 promote proliferation by suppressing targets that normally serve to repress proliferative pathways.

## miR-221/222 in Resistance to Apoptotic Stimuli

Overexpression of miR-221/222 serves to protect cancer cells against various forms of apoptotic stimuli, including chemotherapeutics, endocrine therapies, radiotherapy and detached growth conditions. MCF7 cells resistant to cisplatin have increased miR-221/222 expression compared to the wild type cells [47]. Antagonizing miR-221 in pancreatic cell lines causes increased apoptosis and sensitized the cells to gemcitabine [124]. miR-221 and miR-222 are increased in taxol resistant cells, and addition of miR-221 to breast cancer cells results in increased survival in response to paclitaxel treatment [125]. One of the mechanisms through which miR-221/222 repress apoptosis is through direct targeting of pro-apototic genes, such as PUMA [126] and BMF [106].

Her2/neu amplified breast cancers tend to be resistant to endocrine therapy [127, 128]. miR-221/222 are high in breast cancers that are positive for Her2/neu, compared to Her2/neu negative breast cancers, and overexpression of miR-221/222 causes MCF7 cells to become tamoxifen resistant [129]. miR-221/222 directly target $p27^{KIP1}$ [114] and this is one of the mechanisms through which the cells become tamoxifen-resistant. In xenograft tumors that are resistant to tamoxifen, antagonizing miR-222 sensitizes tumors to tamoxifen [130]. miR-221/222 directly target TIMP3, a tissue metalloproteinase inhibitor that normally inhibits tamoxifen resistant tumor growth. In breast cancer cells that have become resistant to tamoxifen through increased miR-221/222 expression, TIMP3 is repressed, and there is a resultant increase in the expression of metalloproteases ADAM17 and ADAM 10, as well as increased growth factor signaling [130].

While MCF7 cells treated with tamoxifen have slightly decreased levels of miR-221/222, cells treated with fulvestrant, either alone or in combination with E2, have increased miR-221/222 expression [131], likely because ER represses miR-221/222 [101], so degradation of ER after fulvestrant binding could relieve repression of miR-221/222. Inhibition of miR-221/222 activity causes decreased proliferation.

Fulvestrant resistance is explained in part by the downregulation of p27$^{KIP1}$ and p57$^{KIP2}$ [111, 112], and ER [100, 101]. Increased β-catenin contributes to fulvestrant resistance and E2 independent growth [132]. Cells overexpressing miR-221/222 have increased nuclear β-catenin, corresponding to increased β-catenin-mediated transcriptional activity. TGF-β1 blocks proliferation in wild type MCF7s, but not the fulvestrant resistant cells [133, 134]. However, overexpression of miR-221 or miR-222 in wild type cells increases survival in response to TGF-β1, and antagonizing these miRNAs in resistant cells increases sensitivity [131]. Therefore, it is possible that miR-221/222 are involved in switching the effect of TGF-β signaling from tumor suppressive to tumor promotional. The genes and phenotypes regulated by miR-221/222 are depicted in Fig. 2.

## Regulation of miR-221/222

There is a negative feedback loop between miR-221/222 and ERα. miR-221/222 directly bind to and down-regulate ERα, while ERα binds to estrogen response elements in the promoter of miR-221/222 and represses transcription [101]. Other transcriptional repressors of miR-221/222 function in a cell-type specific manner. For example, in AML cells, the AML1 protein binds to the promoter of miR-221/222 and represses transcription [135]. In melanoma cells, a transcriptional repressor, PLZF (promyelocytic leukemia zinc finger) binds to the promoter of miR-221/222 [136].

FOSL1 (Fra-1) is part of the AP-1 transcription complex and promotes invasiveness and metastatic potential of breast cancers [137–139]. FOSL1 binds an AP-1 site upstream of miR-221/222 and promotes transcription [20]. Activation of the RAS/RAF/MEK pathway increased expression of miR-221/222 in basal breast cancer cells via FOSL1 [20], and activation of the MAPK pathway also increases miR-221/222 expression [D. El-Ashry, Personal Communication].

## Interplay Between the miR-200 and miR-221 Families

Perhaps the most convincing evidence that these two families play an important role in epithelial plasticity in breast cancer comes from the White lab, in a study where breast cancer cells were forced to undergo EMT by being grown in mammosphere conditions. The resulting cells had decreased miR-200, and increased miR-221/222 [107]. Collectively, as described above, these two families clearly exert opposing effects on polarity, migration and invasion, proliferation, apoptosis, and differentiation.

ZEB1/2 transcription factors promote a mesenchymal phenotype by repressing genes involved in polarity. Therefore, ZEB1/2 is detrimental to an epithelial phenotype, and it is essential that these genes remain suppressed in differentiated epithelial cells. While they are most definitely repressed at the promoter level, epithelial cells have evolved an additional layer of protection against their expression, which is miR-200 mediated repression at the post-transcriptional level. Conversely, miR-221/222 promote expression of ZEB2 indirectly through TRPS1, and therefore these miRNAs tend to only be expressed in cells that have undergone EMT [20].

miR-221/222 directly target and repress Dicer, while miR-200c increases Dicer by a yet to be identified mechanism [21]. miR-221/222 are more highly expressed in TNBC [21, 100]. miR-103/107 have also been demonstrated to directly target

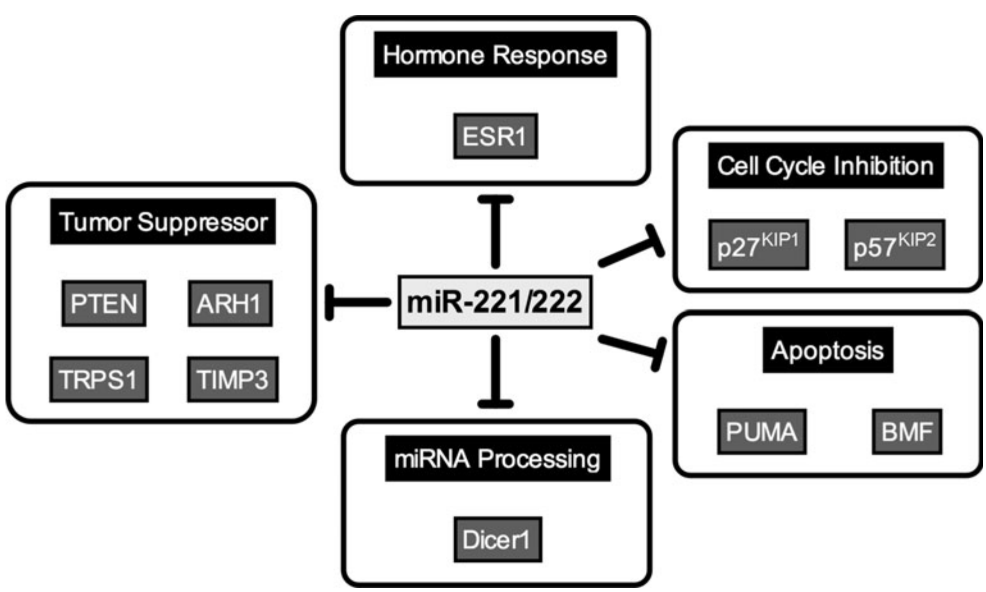


**Fig. 2** Direct targets of miR-221/222. miR-221/222 directly target and down-regulate genes associated with differentiation or tumor suppression. References are included in the text

Dicer [140]; however, an inverse correlation between these miRNA and Dicer has not been as well documented as it has for miR-221/222 which are high in tumors in which Dicer levels are low (TNBC). Thus, miR-221/222 may keep Dicer levels low in poorly differentiated breast cancers [21]. Since Dicer is required for the maturation of most miRNAs, this may explain why overall miRNA expression is lower in TNBC than luminal. Dicer is often low in cancers that have undergone EMT [53]. Dicer is clearly lower in TNBC than adjacent normal breast epithelial cells, while in luminal A breast cancers the difference between tumor and normal is much less dramatic (Fig. 3). Interestingly, TAp63 was recently discovered to suppress metastasis by positively regulating Dicer [141]. It is possible that miR-200c increases Dicer through its ability to repress ZEB1, which upregulates deltaNp63 [142], a dominant negative inhibitor of TAp63. Consequently, the miR-221 and miR-200 families may control the global miRNA landscape in normal and cancerous cells by dueling for control of Dicer. Much remains to be explored to fully determine how the influence of these miRNA families over Dicer might control motility and metastasis in normal development and cancer.

## Conclusions

The role of miRNAs in tumorigenesis and the power they wield with respect to phenotypic control and tumor behavior is just beginning to be understood. In this review we focus on two of the most dysregulated miRNA families in breast cancer, the miR-200 and miR-221 families. The miR-200 family serves to protect the epithelial phenotype, while simultaneously suppressing EMT and tumorigenesis. The miR-200 family protects against migration/invasion, anoikis and therapeutic resistance, and stem cell-like properties. Conversely, miR-221/222 promote a mesenchymal-like phenotype, and support tumorigenesis. Expression of miR-221/222 inhibits tumor suppressors and genes involved in apoptosis, cell cycle inhibition, and miRNA processing. Both miRNA families impinge on two important pathways: EMT through ZEB1/2, and miRNA processing through Dicer.

These two miRNA families promote dueling phenotypes, thus they are coordinately regulated during cellular transformations such as EMT and MET (Fig. 4). During oncogenic EMT the miR-200 family is strongly down-regulated, while miR-221/222 are highly up-regulated and the reverse is true during MET. This suggests that not only is each miRNA family important for induction of their respective phenotypes, but that the coordinated inverse regulation of these families is required to fully achieve an epithelial or mesenchymal phenotype and associated functional properties. In contrast to their now quite evident role in breast cancer, to date, these miRNA families have not been specifically examined in the normal human breast or mouse mammary gland, although some of their identified targets are clearly relevant in the normal gland.

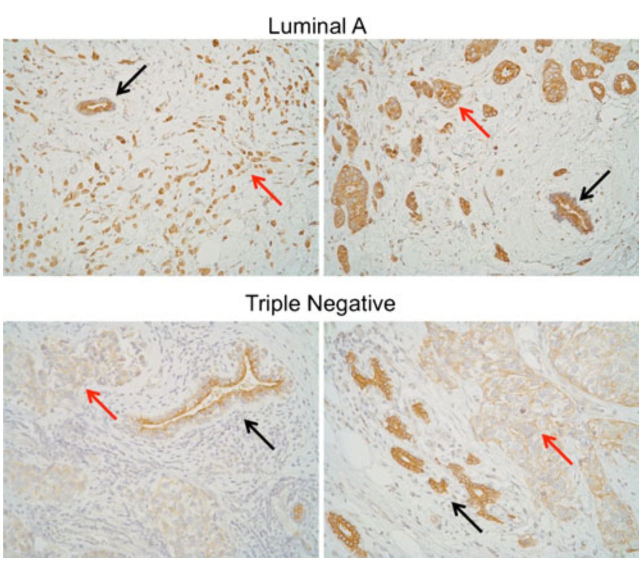


**Fig. 3** Dicer protein expression in luminal A and triple negative breast cancer. Formalin-fixed paraffin embedded sections of human breast cancers were stained for Dicer using ab5818 polyclonal antibody (Abcam, Cambridge, MA). Two representative cases each of luminal and triple negative are shown in which adjacent normal glands are present in the same field of vision (top=luminal, bottom=triple negative) with adjacent normal tissue. Red arrows=tumor, black arrows=normal, 200X

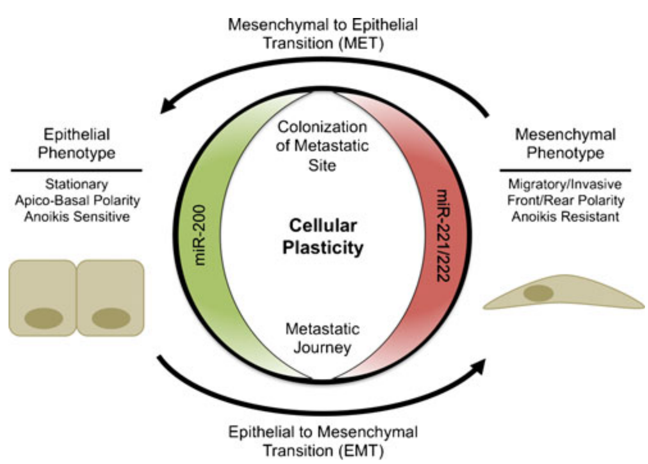


**Fig. 4** Phenotypic consequences of miR-200 or miR-221/222 expression. In addition to the roles of miR-200 and miR-221/222 in protecting the epithelial or mesenchymal phenotype, respectively, they are also actively regulated during EMT and MET. Green indicates expression of the miRNA is associated with a less aggressive, epithelial phenotype, while red indicates the miRNA is associated with aggressive behavior

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RESEARCH ARTICLE Open Access

# Targets of miR-200c mediate suppression of cell motility and anoikis resistance

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## Abstract

**Introduction:** miR-200c and other members of the miR-200 family promote epithelial identity by directly targeting ZEB1 and ZEB2, which repress E-cadherin and other genes involved in polarity. Loss of miR-200c is often observed in carcinoma cells that have undergone epithelial to mesenchymal transition (EMT). Restoration of miR-200c to such cells leads to a reduction in stem cell-like characteristics, reduced migration and invasion, and increased sensitivity to taxanes. Here we investigate the functional role of novel targets of miR-200c in the aggressive behavior of breast and endometrial cancer cells.

**Methods:** Putative target genes of miR-200c identified by microarray profiling were validated as direct targets using dual luciferase reporter assays. Following restoration of miR-200c to triple negative breast cancer and type 2 endometrial cancer cell lines that had undergone EMT, levels of endogenous target mRNA and respective protein products were measured. Migration and sensitivity to anoikis were determined using wound healing assays or cell-death ELISAs and viability assays respectively.

**Results:** We found that restoration of miR-200c suppresses anoikis resistance, a novel function for this influential miRNA. We identified novel targets of miR-200c, including genes encoding fibronectin 1 (FN1), moesin (MSN), neurotrophic tyrosine receptor kinase type 2 (NTRK2 or TrkB), leptin receptor (LEPR), and Rho GTPase activating protein 19 (ARHGAP19). These targets all encode proteins normally expressed in cells of mesenchymal or neuronal origin; however, in carcinoma cells that lack miR-200c they become aberrantly expressed and contribute to the EMT phenotype and aggressive behavior. We showed that these targets are inhibited upon restoration of miR-200c to aggressive breast and endometrial cancer cells. We demonstrated that inhibition of MSN and/or FN1 is sufficient to mediate the ability of miR-200c to suppress cell migration. Lastly, we showed that targeting of *TrkB* mediates the ability of miR-200c to restore anoikis sensitivity.

**Conclusions:** miR-200c maintains the epithelial phenotype not only by targeting ZEB1/2, which usually facilitates restoration of E-cadherin expression, but also by actively repressing a program of mesenchymal and neuronal genes involved in cell motility and anoikis resistance.

## Introduction

Epithelial to mesenchymal transition (EMT) occurs during development as it is required for formation of the neural crest and palate, among other processes [1,2]. In cancer it is a pathological event associated with tumor progression and is thought to influence certain steps in the metastatic cascade, thereby contributing to the metastatic potential of carcinomas. Specifically, EMT likely contributes to the ability of carcinoma cells to invade through basement membrane and stroma and to intravasate into blood and lymph vessels [3-5]. The process of EMT is regulated by several transcription factors, including Twist, SNAIL, SLUG, ZEB1 (zinc finger E-box binding homeobox 1) and the closely related SIP1 (ZEB2), as reviewed in [6], which are transcriptional repressors of *E-cadherin*.

The miR-200 family of miRNAs, which includes miR-200c and miR-141 on chromosome 12 and miR-200a/b and miR-429 on chromosome 1, directly targets ZEB1 and ZEB2 [7-10]. Restoring miR-200c to aggressive breast, endometrial and ovarian cancer cells substantially

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decreases migration and invasion [9-13]. Since ZEB1 represses E-cadherin [14] and other genes involved in polarity [15], the reduction in migratory and invasive capacity observed when miR-200c is restored to cancer cells is widely thought to be due to the ability of miR-200c to target and repress ZEB1/2 which, in most cases, allows E-cadherin to be re-expressed. However, even in cell lines in which E-cadherin is not restored, miR-200c still dramatically reduces migration and invasion [11], implying that additional miR-200c targets can facilitate its ability to suppress cell motility.

We identify and confirm novel direct targets of miR-200c, including the genes encoding fibronectin 1 (FN1), moesin (MSN), neurotrophic tyrosine receptor kinase type 2 (NTRK2 or TrkB), leptin receptor (LEPR), and Rho GTPase activating protein 19 (ARHGAP19). These targets are all genes usually expressed in cells of mesenchymal or neuronal origin. However, in carcinoma cells that lack miR-200c, repression of these genes is compromised and they are allowed to be translated and contribute to an EMT phenotype and aggressive behavior. Here we show that *MSN* and *FN1* are direct targets of miR-200c that contribute to the ability of miR-200c to suppress migration. We also identify a completely novel role for miR-200c - the ability to reverse anoikis resistance and we further pinpoint *TrkB* as the direct target that mediates this effect. Anoikis resistance is an important, yet understudied, step in the metastatic cascade.

## Materials and methods

### Cell culture

Hec50 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. AN3CA cells and Ishikawa cells were grown in MEM with 5% FBS, nonessential amino acids (NEAA), penicillin, streptomycin and 1 nM insulin. MCF-7 cells were grown in DMEM with 10% FBS, and 2 mM L-glutamine. MDA-MB-231 cells were grown in MEM with 5% FBS, HEPES, NEAA, 2 mM L-glutamine, penicillin, streptomycin, and insulin. BT549 cells were grown in RPMI supplemented with 10% FBS and insulin. All cells were grown in a 37°C incubator with 5% $CO_2$. Cell line identities were authenticated by isolating genomic DNA using ZR genomic DNAII kit (Zymo Research, Irvine, CA, USA) and DNA profiling multiplex PCR was performed using the Identifiler Kit (Applied Biosystems, Carlsbad, CA, USA) in the UC Cancer Center DNA Sequencing and Analysis Core.

### Transfection

miR-200c (miRNA mimic) or scrambled negative control (Ambion, Austin, TX, USA) at a concentration of 50 nM were incubated with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in culture medium per the manufacturer's instructions before addition to cells. Cells were incubated at 37°C for 24 hrs before replacement of medium.

### DNA and shRNA constructs

pEGP-MSN (created by Stephen Shaw, National Institutes of Health, purchased from Addgene plasmid 20671, Cambridge, MA, USA) [16]. *FN1* was subcloned from pCR-XL-TOPO-FN1 (Open Biosystems, Catalog number MHS4426-99240322, Huntsville, AL, USA) into pcDNA3.1 (Invitrogen). *TrkB* was subcloned from pBabe-TrkB (a gift from D. Peeper) into pcDNA3.1.

### Microarray analysis

Expression profiling was performed on Hec50 cells transfected as described above and statistical analysis was performed as described previously [12]. Array data have been provided to GEO, accession GSE25332. The heatmap was generated using GeneSpring GX 11 (Agilent, Santa Clara, CA, USA) and shows genes that are statistically significantly down-regulated by at least 1.5-fold in the miR-200c treated samples as compared to either the mock or scrambled control or both, and are predicted to be direct targets of miR-200c. Target site predictions were taken from TargetScan [17], http://microRNA.org[18], PicTar [19] and MicroCosm [20].

### Luciferase assays

A section of the 3' untranslated region (UTR) of each target containing the putative binding site(s) for miR-200c was amplified by PCR from HeLa genomic DNA using the primers listed in Table S1 in Additional file 1. Fragments were cloned into the 3' UTR of a firefly luciferase reporter vector (pMIR-REPORT, Ambion) using HindIII and SpeI. Mutations in the miR-200c binding sites were generated by PCR directed mutagenesis. Mutation primers are listed in Table S1 in Additional file 1 and introduced mutations are in bold and shown above the mRNA in each figure. 3' UTR sequences and mutations were verified by sequencing. Hec50 cells (15,000 per well) plated in a 96-well plate were mock transfected, transfected with negative control, 50 nM miR-200c, 50 nM miR-200c antagomiR (Dharmacon, Lafayette, CO, USA)) alone (α200c) or in conjunction with miR-200c (α200c + 200c). After 24 hrs, the firefly reporter plasmid (196 ng) and a *Renilla* luciferase normalization plasmid pRL-SV40 (4 ng) were introduced using Lipofectamine 2000. Cells were harvested 48 hrs later for analysis using the Dual Luciferase Reporter assay system (Promega, Madison, WI, USA)).

### Real-time reverse transcription-PCR

RNA was harvested from cells using Trizol (Invitrogen) and treated with DNase 1 (Invitrogen) for 15 minutes at room temperature. RNA was reverse transcribed into cDNA in a reaction containing reaction buffer, 10 mM DTT, 1 mM dNTPs, RNase inhibitor (Applied Biosystems), 250 ng random hexamers, and 200 units of MuLV-RT (Applied Biosystems). For normalization, real-time reverse transcription-PCR (RT-PCR) was performed on the cDNA using eukaryotic 18S rRNA endogenous control primers and FAM-MGB probe (Applied Biosystems). TaqMan MicroRNA Reverse Transcription kit was used to generate cDNA for real-time RT-PCR reaction in conjunction with a miR-200c specific primer and probe (Applied Biosystems, assay ID 002300). The reverse transcription primer for miR-200c is a hairpin primer specific to the mature miRNA and will not bind to the precursor molecules. For validation of the microarray data, SYBR Green real-time RT-PCR was performed using primers specific for each target (primers listed in Table S1 in Additional file 1). To avoid the possibility of amplification artifacts, PCR products for all SYBR Green primer pairs were verified to produce single products by agarose electrophoresis and high resolution melt curve. The relative mRNA or miRNA levels were calculated using the comparative Ct method ($\Delta\Delta$Ct). Briefly, the Ct (cycle threshold) values for the rRNA or actin were subtracted from Ct values of the target gene to achieve the $\Delta$Ct value. The $2^{-\Delta Ct}$ was calculated for each sample and then each of the values was divided by a control sample to achieve the relative mRNA or miRNA levels ($\Delta\Delta$Ct).

### Immunoblot analysis

Whole-cell protein extracts prepared in RIPA lysis buffer, equalized to 50 μg by Bradford protein assay (Bio-Rad, Hercules, CA, USA), separated by SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes. For chemiluminecent detection, membranes were blocked in 5% milk in TBS-T and probed overnight at 4°C with primary antibodies. Primary antibodies used were ZEB1 (rabbit polyclonal from Dr. Doug Darling, University of Louisville, Louisville, KY, USA; 1:1,500 dilution), E-cadherin (clone NCH-38 from DAKO, Carpinteria, CA, USA; 1 μg/mL), fibronectin (BD Biosciences, Franklin Lakes, NJ, USA, clone 10/Fibronectin, 1:5000), moesin (Abcam, Cambridge, MA, USA, clone EP1863Y, 1:10,000), ERM (Cell Signaling, Danver, MA, USA, #3142, 1:1000), TrkB (Santa Cruz Biotechnology, Santa Cruz, CA, USA, H-181, #sc8316, 1:200) and $\alpha$-tubulin (Sigma-Aldrich, St. Louis, MO, USA, clone B-5-1-2, 1:30,000). After incubation with appropriate secondary antibody, results were detected using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Waltham, MA, USA). For fluorescent detection, membranes were blocked in 3% BSA (Sigma-Aldrich) in TBS-T and probed overnight at 4°C with primary antibodies. Goat anti-rabbit conjugated to Alexa Fluor 660 (Invitrogen, 1:5,000) and goat anti mouse conjugated to Alexa Fluor 660 (Invitrogen, 1:5,000) were used as appropriate and signal was detected by Odyssey (LI-COR, Lincoln, NE, USA).

### Wound healing assay

Cells were transfected with miR-200c and controls as before and 24 hrs later transfected with vectors. Cells were then plated in six-well plates, allowed to adhere and grow to confluency. Cells were then treated for two hours with 10 μg/mL mitomycin C (Fisher Scientific, Pittsburgh, PA, USA). Wounds were made using a p20 pipet tip and cells were given 24 hrs (Hec50 and BT549) or 48 hrs (AN3CA) to migrate into wounds. Cells were stained with 0.05% crystal violet in 6% glutaraldehyde for one hour, rinsed repeatedly with water, mounted and imaged. For each condition five representative images were obtained for quantitation. Quantitation was performed by first thresholding the images to differentiate between cells (black) and background (white), determining the number of black pixels and the number of white pixels and then calculating the percentage of the image covered by cells.

### Anoikis assay (cell viability and cell death ELISA)

Poly-hydroxyethyl methacrylate (poly-HEMA, Sigma-Aldrich) was reconstituted in 95% ethanol to a concentration of 12 mg/mL. To prepare poly-HEMA coated plates, 0.5 mL of 12 mg/mL solution was added to each well of a 24-well plate and allowed to dry overnight in a laminar flow tissue culture hood. Cells were transfected as before. Twenty-four hours after transfection 50,000 cells were plated in triplicate in poly-HEMA coated 24-well plates using regular culture medium. For cell viability assay, at 4 and 24 hrs after addition to poly-HEMA coated plates, viable and dead cells were stained with trypan blue and counted using the ViCell cell counter (Beckman-Coulter, Brea, CA, USA). For cell death ELISA assay (Roche, San Francisco, CA, USA) cells were plated as before, but the medium was collected at 2, 4, 8, 24 and 48 hrs post plating. Each sample was pelleted, lysed and then frozen so that all samples could be read together at 405 nm and 490 nm (reference wavelength). The assay detects fragmented mono and oligonucleosomes in lysed cells by first binding histones with a biotinylated antibody which is bound to a streptavidin-coated plate. Samples are then bound by an HRP labeled anti-DNA antibody and color is developed by using an ABTS substrate.

## Results

### Restoration of miR-200c decreases non-epithelial, EMT associated genes

We utilize breast and endometrial cancer cell lines in which we have previously characterized miR-200c levels as well as expression of classic epithelial and mesenchymal markers [11,12]. The BT549 and MDA-MB-231 cell lines are triple negative breast cancer (TNBC) cell lines, which lack expression of estrogen receptor alpha (ESR1), progesterone receptors, and HER2/neu. The TNBC lines lack E-cadherin and express the mesenchymal markers N-cadherin and vimentin and, therefore, exhibit an EMT phenotype. In contrast, MCF7 cells represent the luminal A subtype of breast cancer, which retains epithelial markers including ESR1 and E-cadherin. The Hec50 and AN3CA cell lines represent aggressive type 2 endometrial cancers that have lost epithelial markers including E-cadherin and ESR1 and gained mesenchymal markers such as N-cadherin and vimentin, indicative of EMT. In contrast, Ishikawa cells represent the less aggressive type 1 endometrial cancer, which retains epithelial markers and does not express mesenchymal markers. Transfection of miR-200c mimic into the dedifferentiated breast and endometrial cancer lines (BT549, MDA-MB-231, Hec50 and AN3CA) results in levels of mature miR-200c comparable to endogenous levels in the more well-differentiated breast and endometrial cancer lines (MCF7 and Ishikawa) (Figure 1a). These results indicate that experiments performed using this concentration of mimic result in miR-200c levels comparable to those observed in cell lines that have not undergone EMT.

By microarray expression profiling, we previously identified genes significantly altered upon restoration of miR-200c to Hec50 cells [12]. Figure 1b is a heatmap of genes known to be involved in EMT that are statistically significantly decreased at least 1.5-fold upon restoration of miR-200c and are bioinformatically predicted to be targets of miR-200c. The heatmap additionally depicts miR-200c targets identified by others such as *ZEB1* and 2 [8,9], cofilin (*CFL1*) [9] and *WAVE3* [21]. In total we identified 74 genes that change more than 1.5-fold and are predicted by two of four target prediction programs to be direct targets of miR-200c Figure S1 in Additional file 1. Of these genes, 68 (92%) are repressed and 6 (8%) are up-regulated when miR-200c is restored. Initial validation of several of the targets with known involvement

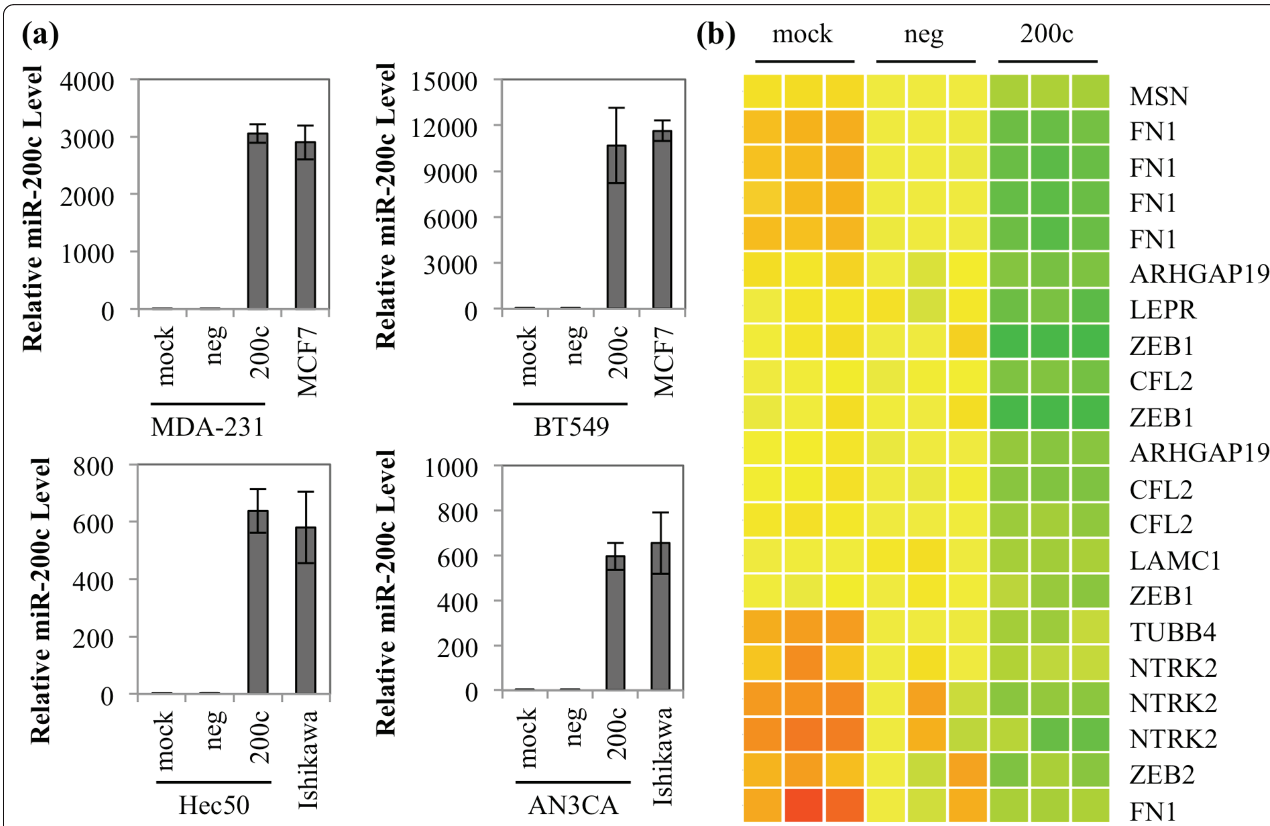


**Figure 1 Restoration of miR-200c decreases EMT associated genes**. **(a)** Cells were treated with transfection reagent only (mock), scrambled negative control (neg) or miR-200c mimic (200c). RNA was harvested after 72 hrs and qRT-PCR was performed for miR-200c. Samples are normalized to 18S rRNA and presented relative to mock. Columns, mean of three biological replicates, bars, standard deviation of the mean. **(b)** Heatmap of genes statistically significantly affected by restoration of miR-200c to Hec50 cells and bioinformatically predicted to be targeted by miR-200c.

in EMT revealed that they are down-regulated at the message level in one or more of our model cell lines Figure S2 in Additional file 1. Based on these findings, we selected *FN1*, *MSN*, *ARHGAP19*, *LEPR* and *TrkB* (*NTRK2* on the heatmap) to experimentally confirm as direct targets of miR-200c.

### Breast and endometrial cancer cell lines that have undergone EMT and express ZEB1, also express FN1, MSN or both

Since there is substantial evidence in the literature for FN1 and MSN being involved in cancer cell migration, we assayed the breast and endometrial cancer cell lines for expression of these proteins (Figure 2). We found that neither the luminal A breast cancer cell line (MCF7) or the type 1 endometrial cancer cell line (Ishikawa) express FN1 or MSN, consistent with their pre-EMT phenotype, indicated by expression of E-cadherin and lack of ZEB1. In contrast, all of the TNBC and type 2 endometrial cancer lines express either one or both of these proteins in addition to ZEB1, supporting the hypothesis that they may play a role in migration in the absence of miR-200c.

### Moesin (*MSN*), a regulator of cortical actin-membrane binding, is directly targeted and down-regulated by miR-200c

MSN connects the actin cytoskeleton and the cell membrane [22] and is strongly up-regulated in cancers with a poor prognosis, including metastatic breast cancer [23], where it contributes to migratory and invasive capacity [24-26]. The 3' UTR of *MSN* contains two putative miR-200c binding sites (Figure 3a) and we cloned the region containing these sites downstream of luciferase. When miR-200c is restored, we observe a 37% decrease in luciferase activity only in the presence of miR-200c and not the controls. To determine the specificity of this down-regulation, we mutated the putative miR-200c binding sites and observe that luciferase activity levels return to levels observed in the absence of miR-200c; thus, miR-200c binding to these sites specifically is required for down-regulation. We also observe that mutating either binding site results in a partial increase in luciferase activity, but only when both sites are mutated is there a full restoration of luciferase activity. Therefore, both binding sites are functional and required for miR-200c to exert its full effect on the *MSN* 3' UTR. When an antagomiR is used to inhibit miR-200c binding to the target sites, luciferase activity is again restored. This indicates that miR-200c specifically is responsible for targeting the *MSN* 3' UTR and the consequent decrease in luciferase activity. Importantly, restoration of miR-200c decreases MSN protein levels (Figure 3b) in two cell lines that express detectable MSN protein, indicating that direct targeting of *MSN* by miR-200c exerts a measurable effect on MSN protein expression.

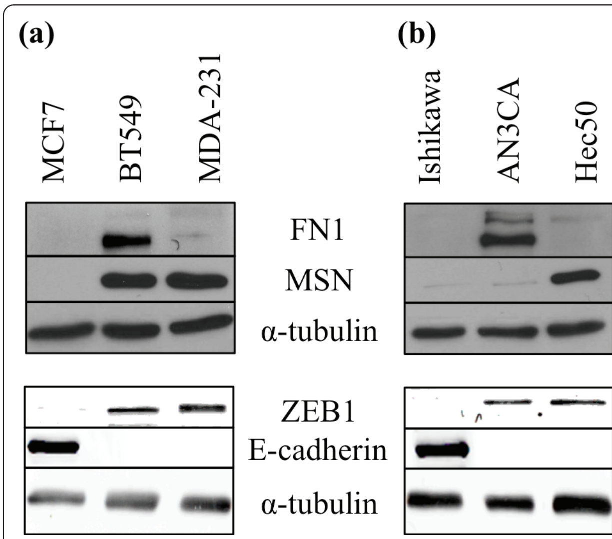


**Figure 2 Breast and endometrial cancer cells can express FN1 and/or MSN**. Breast **(a)** and endometrial **(b)** cancer cell lines analyzed by immunoblot for FN1, MSN, ZEB1, E-cadherin and α-tubulin expression (loading control).

### Down-regulation of MSN contributes to miR-200c mediated suppression of migration

Because miR-200c decreases migration, we next sought to determine the role of MSN in the ability of miR-200c to inhibit migration. Restoration of miR-200c to BT549 and Hec50 cells results in a dramatic decrease in their ability to close a wound as indicated by movement of cells past the initial boundary of the wound (black line) (Figure 4a). BT549 cells display a 41% decrease in migratory ability, while Hec50 cells display a 32% decrease (Figure 4b). The addition of a plasmid encoding MSN lacking its 3' UTR, rendering it untargetable by miR-200c, abolishes the ability of miR-200c to decrease migration (Figure 4a, b) without further increasing the migratory ability of the mock and negative control transfected cells. This indicates that miR-200c targeting of *MSN* can play a critical role in the ability of miR-200c to decrease migration in these cell lines. The levels of MSN protein achieved with the transfection are reasonable (Figure 4c) and do not interfere with the ability of miR-200c to restore E-cadherin in these cell lines.

### The extracellular matrix protein fibronectin 1 (*FN1*) is directly targeted and down-regulated by miR-200c

FN1 is normally expressed by fibroblasts but not epithelial cells, and is a classic marker of the EMT phenotype

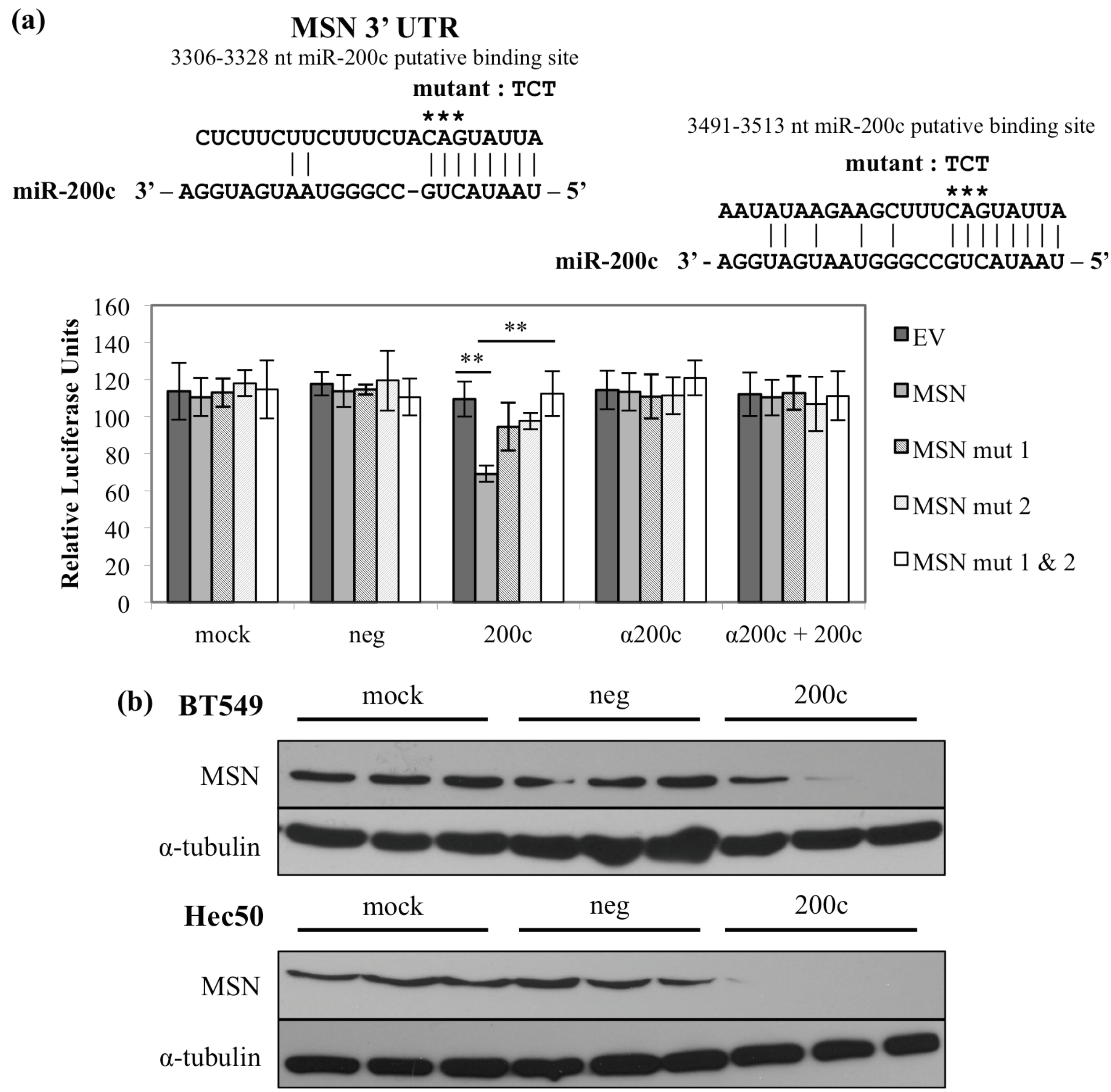


**Figure 3 Moesin (*MSN*), a regulator of cortical actin-membrane binding, is directly targeted and down-regulated by miR-200c**. **(a)** Regions of the 3' UTR where miR-200c is predicted to bind. Hec50 cells treated with transfection reagent only (mock), scrambled negative control (neg), miR-200c mimic (200c), miR-200c antagomiR alone (α200c) or in conjunction with miR-200c (α200c + 200c) and luciferase assay performed. Columns, mean of five replicates, bars, standard deviation of the mean. ANOVA with Tukey-Kramer post-hoc test, ** $P < 0.01$. **(b)** Immunoblot for MSN and α-tubulin (loading control) expression.

and tumorigenicity [27-29]. We [12] and others [8] previously observed a decrease in *FN1* transcript upon restoration of miR-200c and we sought to determine if this is due to direct targeting. Like *MSN*, *FN1* contains two putative miR-200c binding sites in its 3' UTR. When miR-200c is restored, we observe a 76% decrease in luciferase activity only in the presence of miR-200c and not in the controls (Figure 5a). As for *MSN*, mutated constructs show that miR-200c binding to these sites specifically is required for down-regulation and both binding sites are functional and required for miR-200c to exert its full effect on the *FN1* 3' UTR. When an antagomiR is used to inhibit miR-200c binding to the target sites, luciferase activity is again restored. This indicates that miR-200c specifically is responsible for targeting the *FN1* 3' UTR and the consequent

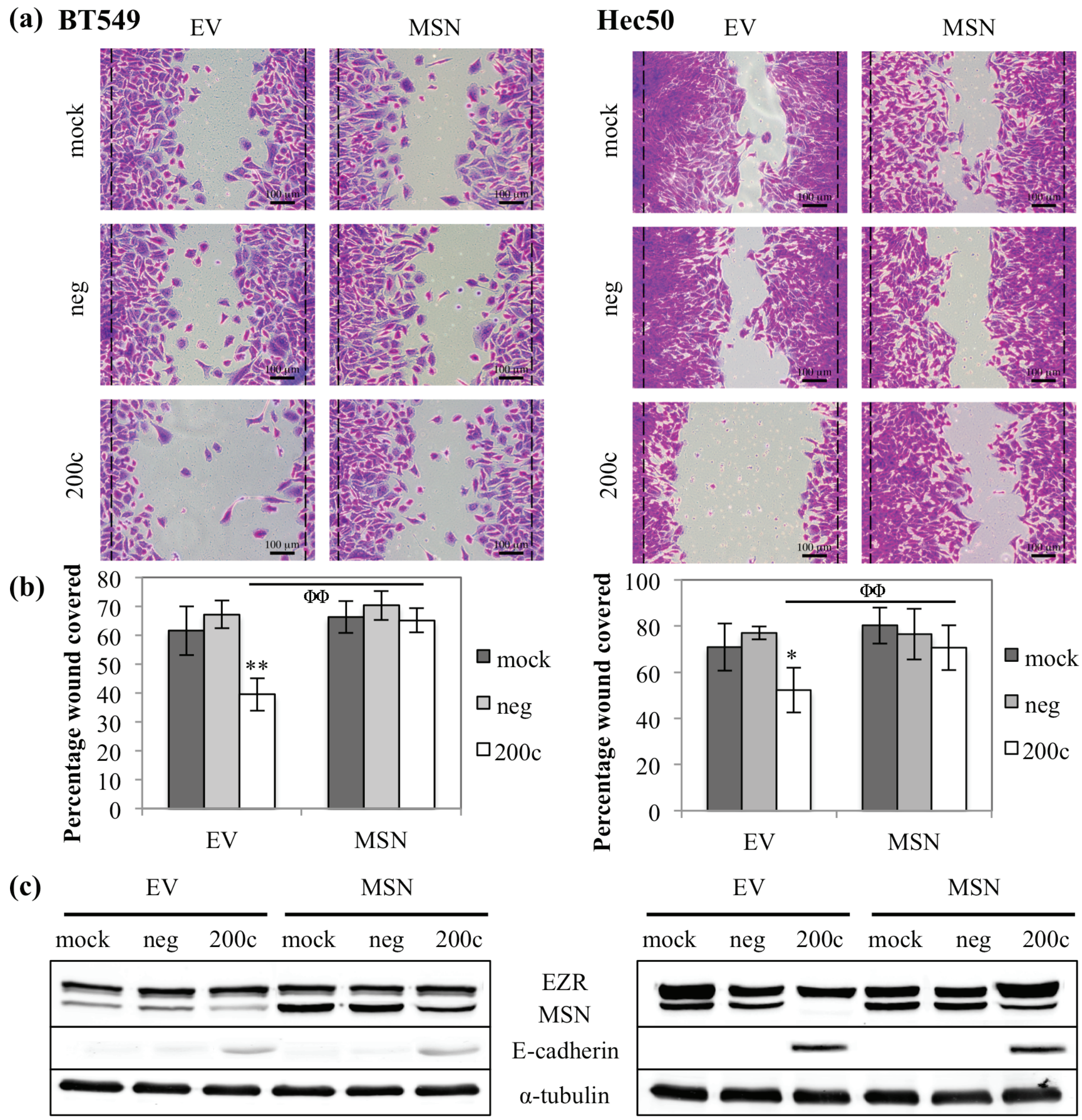


**Figure 4 Down-regulation of MSN contributes to miR-200c mediated suppression of migration**. Cells were transfected with empty vector (EV) or MSN and 24 hrs later with miRNA constructs. BT549 (left) and Hec50 (right) cells were treated with mitomycin C and given 24 hrs to migrate. **(a)** Brightfield images of crystal violet stained cells, dashed black lines indicate edges of the wound immediately after wounding. Scale bars are 100 μm. **(b)** Quantitation of migratory ability of cells. Columns, mean of five replicates, bars, standard deviation of the mean. ANOVA, * $P < 0.05$, ** $P < 0.01$, Tukey-Kramer post-hoc test, ΦΦ $P < 0.01$. **(c)** Immunoblot for MSN, E-cadherin and α-tubulin (loading control).

decrease in luciferase activity. Only the AN3CA and BT549 express detectable protein levels (Figure 2) and restoration of miR-200c to these cell lines dramatically decreases FN1 protein expression (Figure 5b).

### Down-regulation of FN1 contributes to miR-200c mediated suppression of migration

We next sought to determine if FN1 plays a role in miR-200c control of migration. Restoration of miR-200c to BT549 and AN3CA cells again results in a dramatic decrease in migration (Figure 6a), which is abrogated by addition of an untargetable *FN1* plasmid. The BT549 cells exhibit a 43% decrease in migratory ability, while the AN3CA cells decrease 53% (Figure 6b). Thus, down regulation of FN1 is an additional mechanism by which miR-200c suppresses migration in aggressive breast and endometrial cancer cell lines. The levels of FN1 protein achieved with the plasmid are reasonable and do not interfere with the ability of miR-200c to restore E-cadherin expression in the BT549 cell (Figure 6c). The

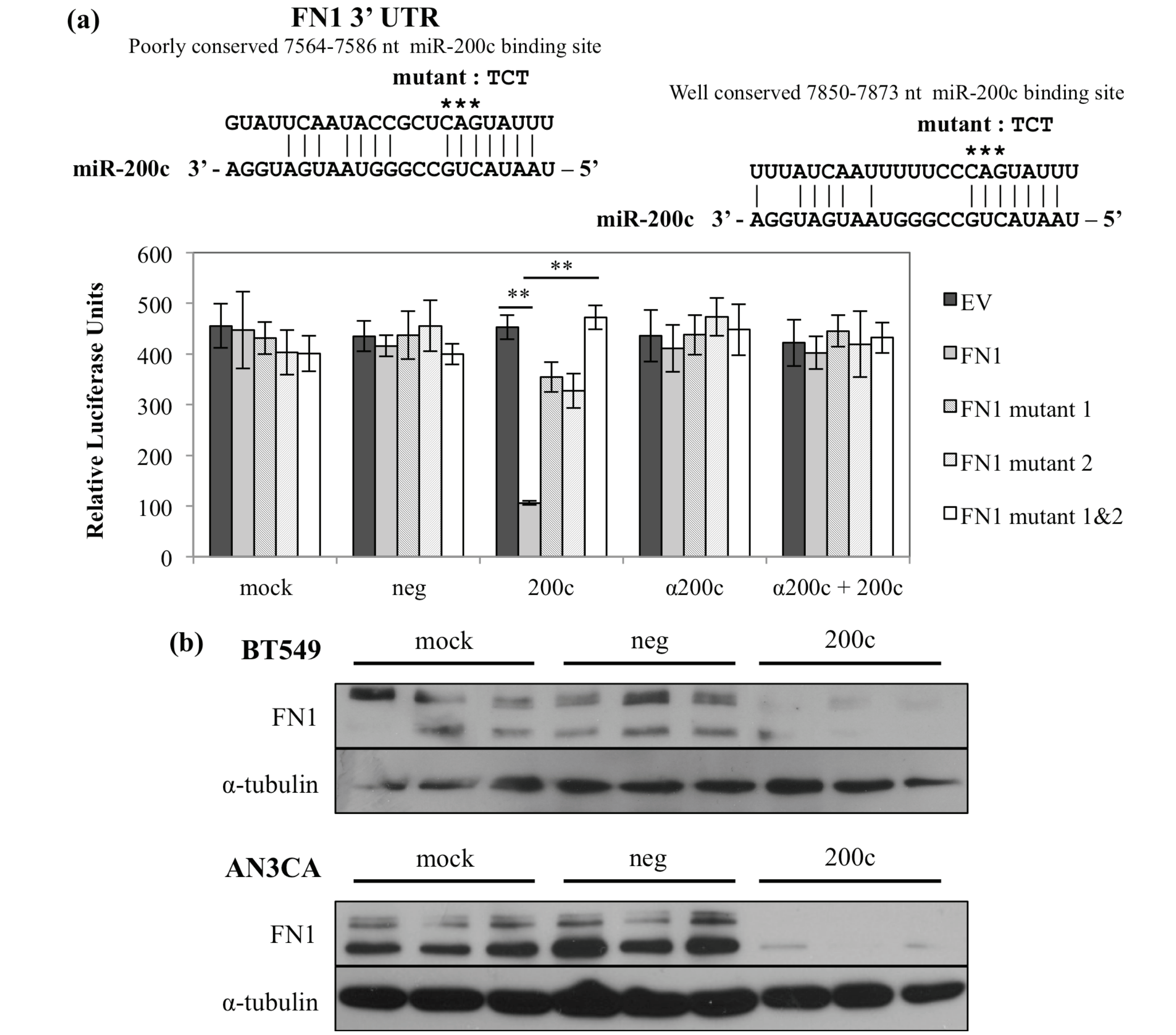


**Figure 5 The extracellular matrix protein fibronectin (*FN1*) is directly targeted and down-regulated by miR-200c**. **(a)** Regions of the 3' UTR where miR-200c is predicted to bind. Hec50 cells treated and luciferase assay performed. Columns, mean of five replicates, bars, standard deviation of the mean. ANOVA with Tukey-Kramer *post-hoc* test, ** $P < 0.01$. **(b)** Immunoblot for FN1 and α-tubulin (loading control) expression.

AN3CA cells do not re-express E-cadherin following restoration of miR-200c.

### The genes encoding Rho GTPase activating protein 19 (*ARHGAP19*) and leptin receptor (*LEPR*) are directly targeted and down-regulated by miR-200c

ARHGAP19 is a GTPase activating protein that has not been well characterized, but is predicted to regulate the activity of Cdc42, RhoA and/or Rac1 [30]. The 3' UTR of *ARHGAP19* contains one putative miR-200c binding site. We demonstrate that restoration of miR-200c causes an 80% reduction in luciferase activity only in the presence of miR-200c and not in the controls (Figure S3 in Additional file 1). LEPR and its ligand leptin are involved in the migration/invasion of trophoblasts [31] and the expression of leptin by mammary epithelial cells has been linked to tumorigenicity [32-34]. We demonstrate that restoration of miR-200c causes a 36% reduction in luciferase activity when the 3' UTR of *LEPR* is placed downstream of luciferase (Figure S4 in Additional file 1).

### The anoikis suppressing neurotrophic receptor tyrosine kinase 2 (*NTRK2* or *TrkB*) is directly targeted and down-regulated by miR-200c

TrkB expression leads to anoikis resistance in several types of cancer, including breast [35-38], and this led us to investigate the regulation of this cell surface receptor

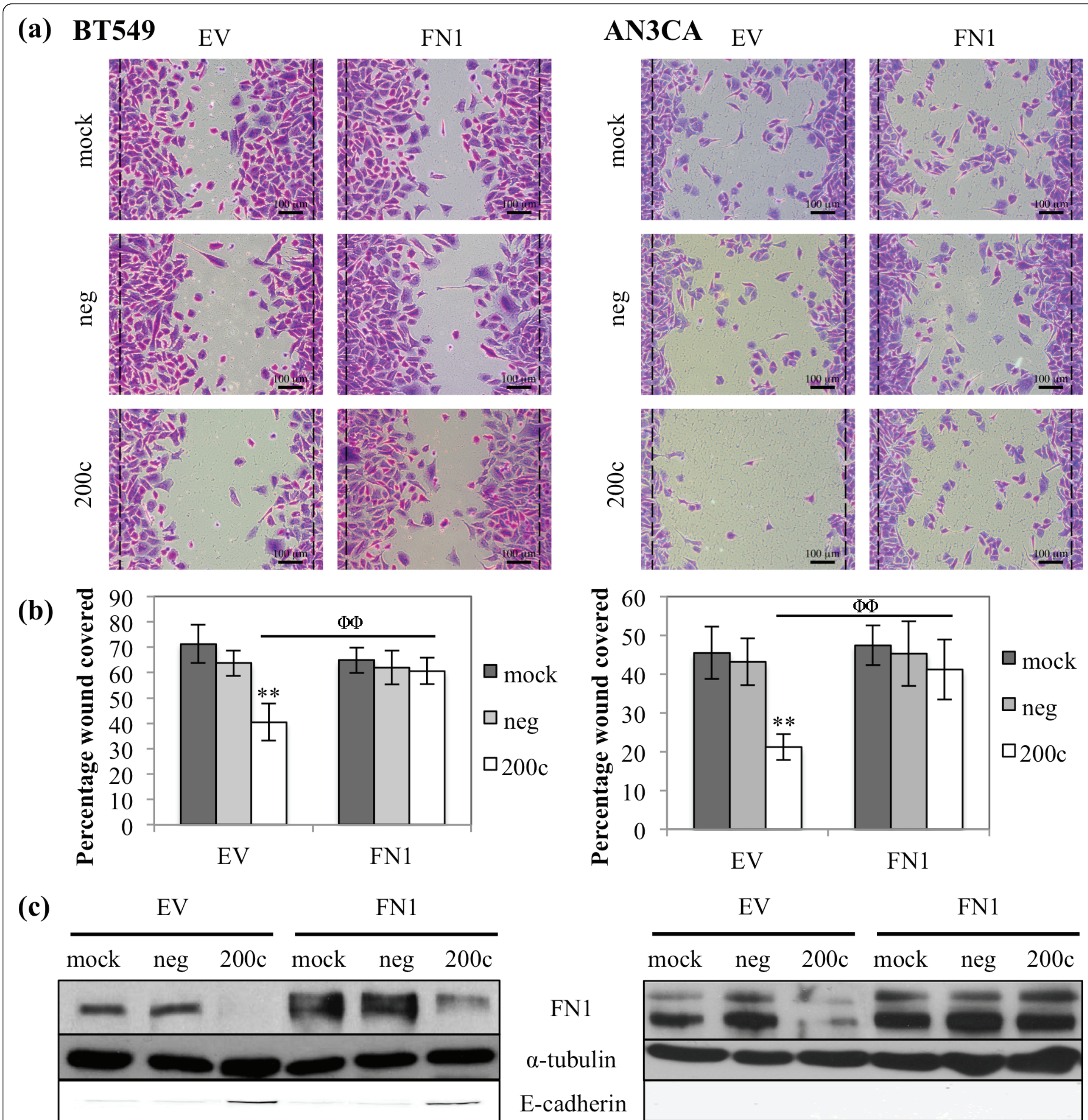


**Figure 6 Down-regulation of FN1 contributes to miR-200c mediated suppression of migration**. Cells were transfected with empty vector (EV) or FN1 and 24 hrs later with miRNA constructs. BT549 (left) and AN3CA (right) cells were treated with mitomycin C and given 24 or 48 hrs, respectively, to migrate. **(a)** Brightfield images of crystal violet stained cells, dashed black lines indicate edges of the wound immediately after wounding. Scale bars are 100 μm. **(b)** Quantitation of migratory ability of cells. Columns, mean of five replicates, bars, standard deviation of the mean. ANOVA, ** $P < 0.01$, Tukey-Kramer post-hoc test, ΦΦ $P < 0.01$. **(c)** Immunoblot for FN1, E-cadherin and α-tubulin (loading control).

by miR-200c. We demonstrate that *TrkB* is a direct target of miR-200c, showing a 55% reduction in luciferase activity (Figure 7a). Luciferase activity is restored following either mutation of the binding site or addition of an antagomiR, indicating that miR-200c binds to the 3' UTR of *TrkB* to downregulate it. Additionally, restoration of miR-200c significantly decreases endogenous TrkB protein in the BT549 and Hec50 cells (Figure 7b).

### miR-200c suppresses anoikis resistance

Given the known role of TrkB in anoikis resistance, we investigated the effect of miR-200c on anoikis by

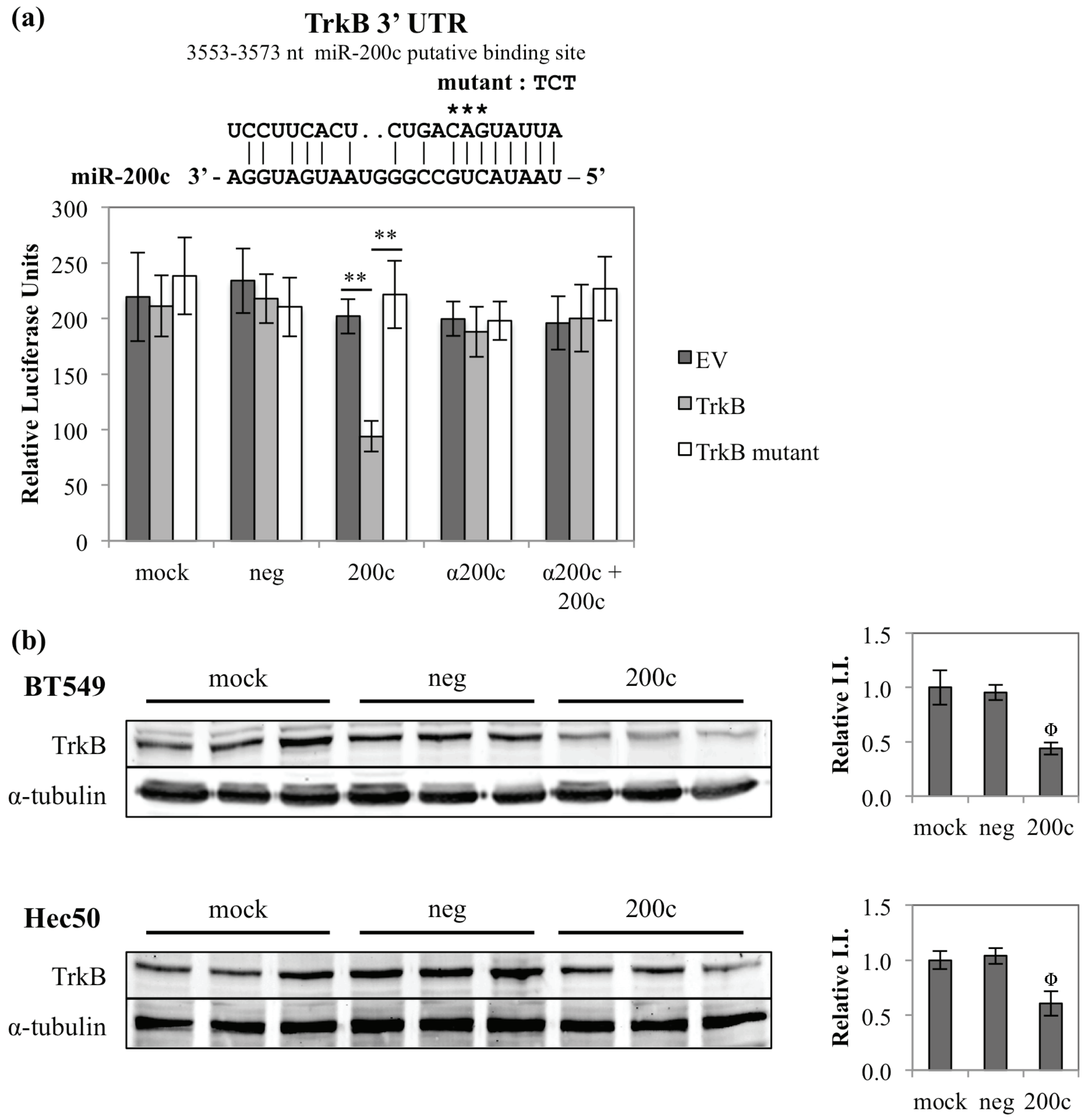


**Figure 7 The anoikis suppressing neurotrophic receptor tyrosine kinase 2 (*NTRK2* or *TrkB*) is directly targeted and down-regulated by miR-200c**. **(a)** The region of the 3' UTR where miR-200c is predicted to bind. Hec50 cells treated and luciferase assay performed. Columns, mean of five replicates, bars, standard deviation of the mean. ANOVA with Tukey-Kramer post-hoc test, ** $P < 0.01$. **(b)** (Right) Immunoblot for TrkB and $\alpha$-tubulin (loading control) expression. (Left) Quantitation of TrkB integrated intensity (I.I.), normalized to $\alpha$-tubulin and presented relative to mock. ANOVA, $\Phi$ $P < 0.05$.

performing cell viability assays and cell death ELISAs. In these assays the cells are plated on poly-HEMA coated plates, which prevents them from adhering. The cells are forced to float in suspension for the times indicated before being harvested for analysis. Cell viability was determined by trypan blue exclusion and shows that restoration of miR-200c significantly decreases viability as quickly as 24 hrs in suspension (Figure 8a). In the cell death ELISAs, restoration of miR-200c results in an increase in fragmented nucleosomes, indicating an increase in apoptosis in these samples (Figure 8b). Thus, restoration of miR-200c decreases anoikis resistance as

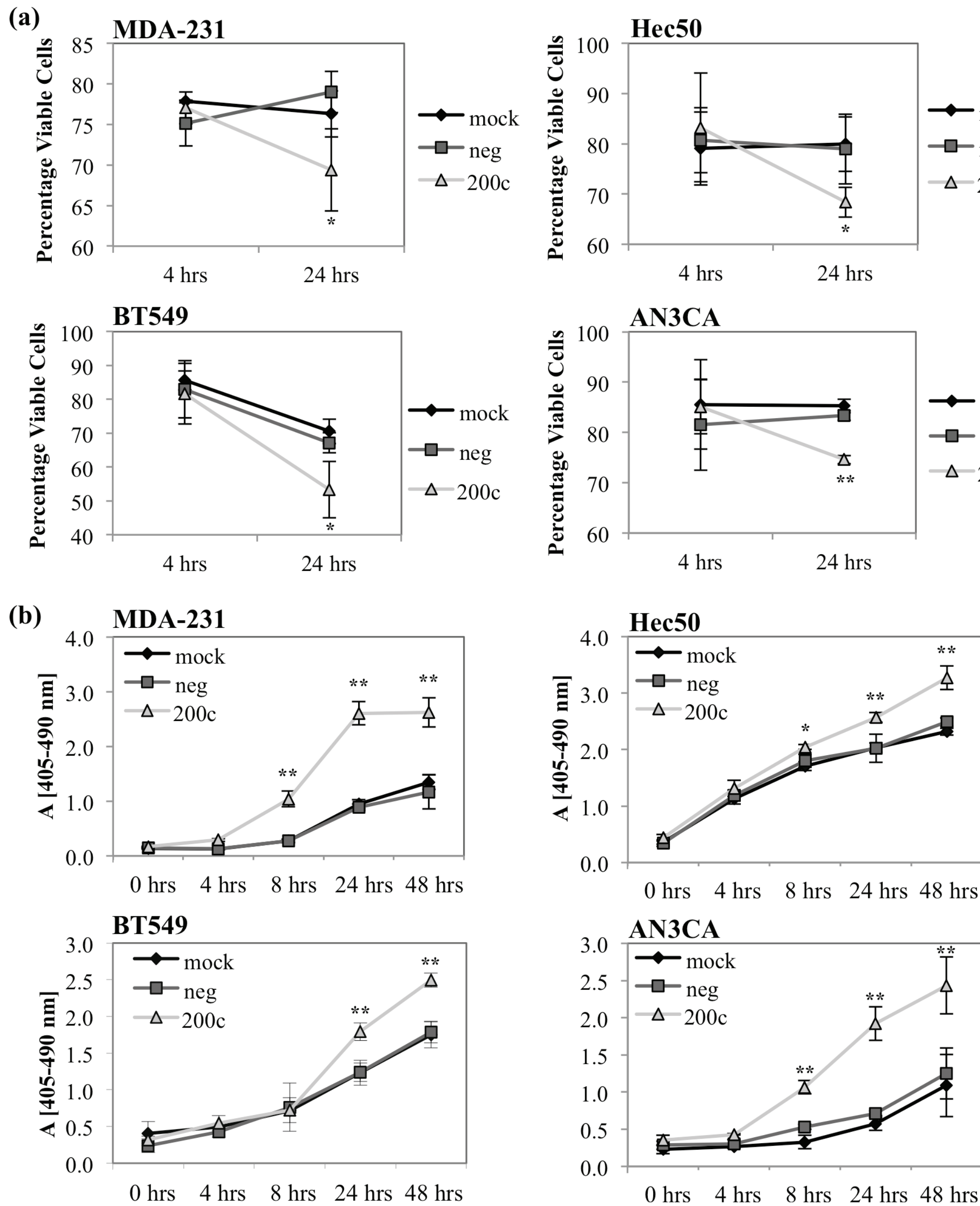


**Figure 8 miR-200c increases sensitivity to anoikis**. Breast (left) and endometrial (right) cancer cells were transfected with miRNA constructs and plated on poly-HEMA coated plates. Cells were collected for viability analysis by trypan blue exclusion **(a)** or apoptosis analysis by cell death ELISA **(b)**. Columns, mean of three biological replicates, bars, standard deviation of the mean. ANOVA, * $P < 0.05$, ** $P < 0.01$.

indicated by a decrease in the viability of suspended cells and concurrent increase in apoptosis.

### Down-regulation of TrkB contributes to miR-200c mediated suppression of anoikis resistance

To determine if targeting of *TrkB* is responsible for the ability of miR-200c to restore sensitivity to anoikis, we used a plasmid encoding *TrkB* lacking the 3' UTR, rendering it untargetable by miR-200c. Restoration of miR-200c enhances sensitivity to anoikis (Figures 8 and 9), but this phenotype is completely reversed in the presence of exogenous, untargetable *TrkB* (Figure 9a, c). However, it is important to note that the addition of exogenous TrkB does not decrease the amount of cell

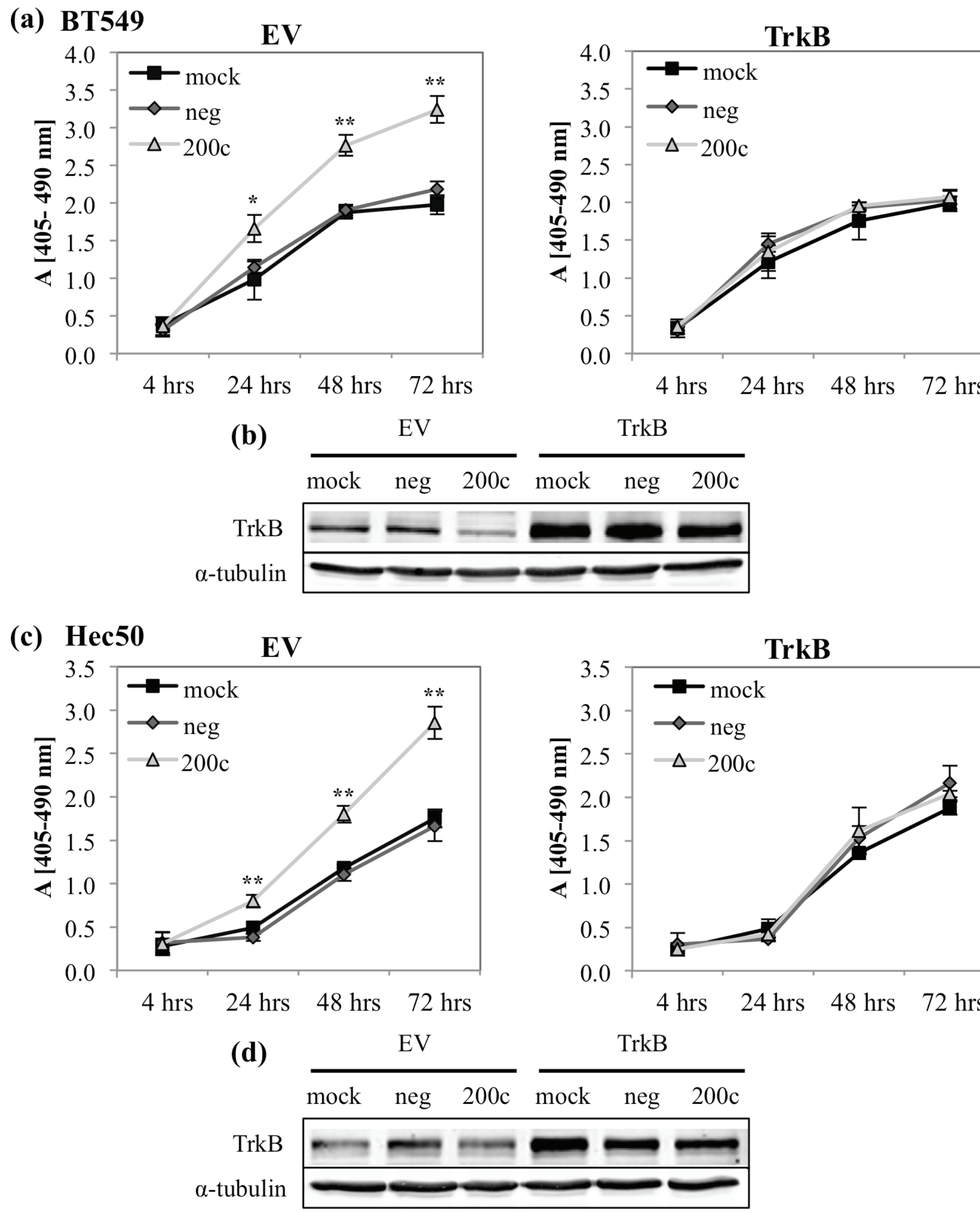


**Figure 9 Down-regulation of TrkB contributes to miR-200c mediated suppression of anoikis resistance**. Cells were transfected with empty vector (EV) (left) or TrkB (right) and 24 hrs with miRNA constructs. Twenty-four hours later cells were plated on poly-HEMA coated plates and cell death ELISA performed at time points indicated **(a)** and **(c)**. Columns, mean of three biological replicates, bars, standard deviation of the mean. ANOVA, * $P < 0.05$, ** $P < 0.01$. **(b)** and **(d)** Immunoblot for TrkB and α-tubulin (loading control).

death in mock or negative control transfected cells. This indicates that miR-200c targeting of *TrkB* plays a critical role in the ability of miR-200c to reverse anoikis resistance.

## Discussion

Progression and metastasis of carcinomas is a multistep process. EMT is thought to aid cancer cells as they invade through basement membrane and stroma, intravasate into blood or lymph vessels, and may also facilitate anoikis resistance, allowing tumor cells to survive the journey to the metastatic site. We sought to identify additional direct targets of miR-200c that mediate its potent effects.

Three of the new direct targets of miR-200c that we identify, *MSN*, *FN1*, and *ARHGAP19*, have been implicated in migration and invasion. MSN localizes to the trailing edge of invasive melanoma cells and disruption of this localization leads to decreased metastasis [25]. MSN expression correlates with poor prognosis in oral squamous cell carcinoma [24] and basal breast cancer [23], a subtype with high risk of metastasis and recurrence. FN1 functions in cell migration through integrin binding [39] and can activate focal adhesion kinase (FAK) leading to increased motility and invasion of carcinoma cells [27,28]. ARHGAP19 is a member of a family of GTPase activating proteins, and other family members, 8, 9, 12 and 15, are expressed in several types of cancer and activate Cdc42, Rac1 or RhoA [40-43], small GTPases required for migration. We demonstrate that *FN1* and *MSN* are, at least in some cell lines, critical targets sufficient to mediate miR-200c's ability to inhibit migration in an *in vitro* wound healing assay. In some cell lines both MSN and FN1 are expressed, and in those cells both MSN and FN1 may contribute to migratory potential, but they are both repressed when miR-200c is restored. In other TNBC cells and type 2 endometrial cancer cells, either MSN or FN1 are expressed but not both. It is possible that even though miR-200c is absent, additional miRNA(s) that target these genes may be retained in some cells, or alternatively, factors that induce these genes at the promoter may be differentially expressed. In some cases ARHGAP19 may additionally contribute to migratory capacity; however, at present there is no antibody available to detect this protein. Loss of miR-200c could permit any of these genes, typically expressed in the more motile mesenchymal or neuronal cell types, to be inappropriately translated and expressed in epithelial cells. Expression of proteins such as MSN that actively contribute to cell motility by promoting front-rear polarity, combined with the loss of E-cadherin (which would decrease cell-cell attachments and reduce apical-basal polarity), may significantly contribute to the invasive capacity of carcinomas.

We demonstrate that restoration of miR-200c leads to a dramatic increase in sensitivity to anoikis (over a 100% increase in anoikis in some cell lines) and identify *TrkB* as a novel direct target of miR-200c. TrkB is a tyrosine kinase cell surface receptor typically expressed on neurons, which can be inappropriately expressed in carcinomas [44]. In breast and ovarian cancer cell lines TrkB induces anoikis resistance [31,33] and can induce EMT through activation of Twist [41]. We previously demonstrated that miR-200c does not affect apoptosis when endometrial cancer cells are attached to plastic, although it does enhance apoptosis induced by taxanes [11,12]. Thus, we conclude that miR-200c specifically enhances anoikis sensitivity, suggesting that restoration of miR-200c could limit the ability of breast and endometrial cancer cells to survive in the bloodstream.

Interestingly, all of the new miR-200c direct targets that we identify in this study (as well as other previously identified targets such as ZEB1/2 and TUBB3) contribute to the designation of this miRNA as a "guardian of the epithelial phenotype" because they are genes typically expressed in cells of mesenchymal or neuronal origin, but not in normal, well-differentiated epithelial cells.

Not all of the target genes that we identify change at the message level upon restoration of miR-200c. For example, although miR-200c directly targets *ARHGAP19* (Figure S3 in Additional file 1), the message is down-regulated by addition of miR-200c in only 3 of 4 cell lines (Figure S2 in Additional file 1). There are several possible explanations for interference between a miRNA and its mRNA target in some cell lines. The miR-200c target site may be mutated or absent due to a shortening of the 3' UTR [46-49] or there may be RNA binding proteins present in particular cell lines that prevent miR-200c from binding [50]. Importantly, for all of the targets that we follow up on in this study (MSN, FN1 and TrkB), protein levels are affected by miR-200c, indicating that it does have an affect on translation of these genes, regardless of whether it also affects degradation of the message.

## Conclusions

In summary, miR-200c inhibits migration and invasion [9-13], stemness [51,52], and chemoresistance [11,12] and we now identify a completely novel role for miR-200c - the ability to reverse anoikis resistance, an important additional step in the metastatic cascade. We identify new targets of miR-200c, which together with previously identified targets, comprise a program of genes normally restricted to cells of mesenchymal or neuronal origin. We specifically pinpoint *MSN* and *FN1* as well as *TrkB* as targets that can respectively mediate the ability of miR-200c to inhibit cell motility and anoikis resistance.

Members of the miR-200 family are down-regulated in breast cancer stem cells and normal mammary gland stem cells [51]. Polycomb complexes facilitate stem cell self-renewal and pluripotency, and both Bmi1, a component of the PRC1 polycomb complex, and Suz12, a component of the PRC2 polycomb complex, have been identified as targets of miR-200 family members [51-53]. It is interesting to speculate as to whether expression of TrkB is involved in the ability of cancer stem cells to resist anoikis.

If feasible, effective *in vivo* delivery of miR-200c could potentially inhibit multiple steps in tumor progression, including tumor formation, cell motility/invasiveness, anoikis resistance and chemoresistance, by virtue of simultaneously repressing multiple, yet specific, targets expressed in carcinoma cells exhibiting an EMT phenotype. Although one *in vivo* study demonstrated that introduction of miR-200c reduced the ability of primary human breast cancer stem cells to form tumors in immune compromised mice [51], further *in vivo* studies will be necessary to specifically isolate the effects of miR-200 on other steps in the metastatic cascade, such as its potential to reverse anoikis resistance.

## Additional material

**Additional file 1: Additional experimental data and the sequences of primers used in cloning and qRT-PCR**.

#### Abbreviations
DMEM: Dulbecco's modified eagle's medium; EMT: epithelial to mesenchymal transition; ESR1: estrogen receptor alpha; FAK: focal adhesion kinase; FBS: fetal bovine serum; FN1: fibronectin 1; LEPR: leptin receptor; MSN: moesin; NEAA: non-essential amino acids; poly-HEMA: poly-hydroxyethyl methacrylate; TNBC: triple negative breast cancer; UTR: untranslated region.

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#### Authors' contributions
ENH performed experimental studies. DRC performed array profiling studies and generated the heatmap in Figure 1. All authors contributed intellectual input towards the design, implementation, and interpretation of results. ENH and JRK drafted the manuscript and all authors read and approved the final manuscript.

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Breast Cancer RESEARCH

EDITORIAL

# miR-200c at the nexus of epithelial–mesenchymal transition, resistance to apoptosis, and the breast cancer stem cell phenotype

Derek C Radisky*

See related research by Howe *et al*., http://breast-cancer-research.com/content/13/2/R45

**Abstract**

Decreased expression of miRNAs of the miR-200 family has been implicated in the growth and metastasis of breast cancer cells. Of this family, miR-200c has garnered particular attention as a consequence of its ability to target ZEB1 and ZEB2, mediators of epithelial–mesenchymal transition. An article in the previous issue of *Breast Cancer Research* identifies additional targets of miR-200c that link increased cancer cell invasiveness, resistance to apoptosis, and induction of breast cancer stem cell characteristics.

miRNAs are a family of small noncoding RNA molecules that downmodulate gene expression through post-transcriptional mechanisms, and individual miRNAs have been found to play critical roles in mammary gland development and breast cancer progression. In the previous issue of *Breast Cancer Research*, Howe and colleagues find that miR-200c plays a broader role in suppression of breast cancer development than had been previously suspected [1]. Members of the miR-200 family had previously been investigated for their ability to inhibit epithelial–mesenchymal transition (EMT), a developmental process in which epithelial cells acquire the migratory, invasive, and apoptosis-resistant properties of mesenchymal cells [2-4]. To date, the most investigated targets of miR-200 family members have been the transcription factors ZEB1 and ZEB2, regulators of EMT that maintain the mesenchymal phenotype by downmodulating expression of E-cadherin as well as other mediators of epithelial cell polarity and function [5]. Previous studies from the Richer laboratory, however, have shown that reintroduction of miR-200c into Hey ovarian cancer cells led to decreased migration and invasion even though E-cadherin was not re-expressed [6], suggesting that miR-200c could also affect invasion through processes independent of the ZEB1/2–E-cadherin axis.

To identify ZEB1-independent mechanisms by which miR-200c could inhibit cell motility, Howe and colleagues used a panel of breast and endometrial cancer cell lines previously identified as miR-200c deficient [1]. MDA-MB-231 and BT549 breast cancer cells and Hec50 and AN3CA endometrial cancer cells express very low levels of miR-200c, high levels of ZEB1, and little E-cadherin; expression of miR-200c in these cells is sufficient to inhibit ZEB1 and increase expression of E-cadherin [1,7]. Analysis of microarray profiles of Hec50 endometrial cells in which miR-200c had been re-expressed revealed several additional genes that were potential targets of miR-200c and which had been previously implicated in cell motility, including the extracellular matrix protein fibronectin 1 and the actin-organizing protein moesin. Luciferase reporter assays were used to show that miR-200c directly targeted the 3’ UTR of these genes. While expression of miR-200c in these cells led to significantly decreased cell motility and re-expression of E-cadherin, further addition of plasmids encoding either fibronectin 1 or moesin that could not be targeted by miR-200c restored cellular migratory ability without affecting E-cadherin expression levels – effectively demonstrating that miR-200c can affect cell motility through both ZEB1/E-cadherin-dependent and ZEB1/E-cadherin-independent pathways.

Another important mesenchymal characteristic that can be acquired through activation of the EMT program in tumor cells is increased ability to tolerate conditions that should trigger apoptotic cell death. Researchers from the Richer laboratory had previously shown that re-expression of miR-200c in breast, endometrial, and ovarian cancer cells led to increased susceptibility to apoptosis induced by microtubule-targeting chemotherapeutic agents [7]. In the present study, Howe and colleagues identify the neurotrophic receptor tyrosine

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kinase 2 (which encodes the protein TrkB) as a specific target of miR-200c that confers resistance to anoikis [1]. Anoikis is the cell death program activated in anchorage-dependent cells upon separation from the extracellular matrix, suppression of which is believed to be a necessary step in development of breast ductal carcinoma *in situ* [8]. TrkB was shown to act as a mediator of anoikis resistance in both BT549 and Hec50 cells, and restoration of miR-200c caused decreased expression of TrkB protein concomitant with increased death of cells cultured on nonadhesive substrata; resistance to anoikis in the miR-200c-expressing cells was specifically regained by expression of a TrkB construct that could not be targeted by miR-200c. These experiments identify a novel and unexpected potential function for miR-200c in blocking tumor progression. Intriguingly, recent studies with rat kidney epithelial cells have found that ZEB1 is a required downstream effector for TrkB-induced anoikis resistance [9], and other investigators have found that the miR-200c target Fas-associated phophatase 1 is involved in resistance to Fas-mediated apoptosis [10], suggesting that miR-200c can also control apoptosis through both ZEB1-dependent and ZEB1-independent processes.

Induction of the EMT program has also been linked with the breast cancer stem cell (CSC) phenotype, characterized by increased malignant potential and resistance to chemotherapeutic agents [11]. A series of recent studies have implicated miR-200 family members and targets in activation and maintenance of the CSC phenotype, as miR-200c is downregulated in breast cancer cells that express CSC markers [12] and re-expression of miR-200 family members can reverse CSC characteristics [12,13]. Furthermore, while ZEB1 and ZEB2 were previously shown to target miR-200 family members in a self-reinforcing feedback loop [3], recent studies have indicated that ZEB1 also inhibits other miRNAs involved in stem cell characteristics, including miR-203 and miR-183 [14]. It is striking that the key characteristics of the CSC phenotype – the ability to grow on nonadhesive substrata and increased cell motility [15] – are both identified in the current study as being directly regulated by miR-200c [1].

An important consideration when evaluating these pathways in cancer cells is that the function of miR-200 in controlling EMT is likely to be highly dependent upon specific characteristics of the developing tumors; indeed, the targets of miR-200c identified by Howe and colleagues are not invariably induced in every cell line with reduced levels of the miRNA [1]. Unlike developmental EMT, which proceeds as an orchestrated program of many different mediators and effectors to induce an organized outcome, the more chaotic tumor micro-environment can stimulate incomplete or transient activation of the EMT program. As activation of even a subset of EMT-associated processes may be sufficient to confer increased motility or resistance to apoptotic stimuli without complete conversion to a mesenchymal cell, it may be necessary for cellular mediators that regulate EMT to be sufficiently flexible to inhibit many different targets – a job that is perhaps particularly well suited for miRNA. Defining the full range of targets through which miR-200c functions as a tumor suppressor could provide insight into how (and why) activation of the EMT program in tumors is linked to apoptosis resistance and the CSC phenotype, a critical question yet to be fully addressed.

**Abbreviations**

CSC, cancer stem cell; EMT, epithelial–mesenchymal transition; miRNA, microRNA; UTR, untranslated region; ZEB, zinc-finger enhancer binding.

**Competing interests**

The author declares that he has no competing interests.

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Review

# Steroid receptors and microRNAs: Relationships revealed

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that serve as post-transcriptional regulators of gene expression. They work predominantly by binding to complementary sequences in target messenger RNA (mRNA) 3′ untranslated regions (UTRs) where they prevent translation or cause degradation of the message. Steroid hormone receptors (SHRs) are ligand-activated transcription factors that regulate genes in steroid responsive tissues. Recent studies demonstrate that SHRs regulate miRNAs, and in turn, miRNAs can regulate SHR expression and function. Mounting evidence indicates that miRNAs are intimately involved with SHRs, as they are with other transcription factors, often in double negative feedback loops. Investigators are just beginning to expose the details of these complex relationships and reveal the extent to which miRNAs are involved with SHRs in normal physiology and the pathobiology of steroid hormone responsive tissues.



**Contents**



## 1. Introduction

MicroRNAs (miRNAs) are 19–25 nt, non-coding RNAs that induce post-transcriptional gene silencing. The first miRNA was discovered in 1993 in the Ambros lab and was involved in *Caenorhabditis elegans* developmental timing [1]. It later became apparent that miRNAs are expressed in all eukaryotic organisms and are involved in almost all cellular processes. In general,

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miRNAs bind to complementary regions in target mRNAs; however, in mammals this is rarely with complete complementarity and there are often mismatches and bulges [2]. The most important determinant for target specificity is the "seed sequence" of the miRNA, comprising nucleotides 2–8 [3]. The majority of target sites described to date are in the 3′ untranslated region (UTR) of the targets [4,5]; however, miRNAs can also bind to the coding region of their targets or even promoter regions of genes [6–9].

The genes encoding miRNAs reside in intergenic regions as independent units or in introns where they are usually co-transcribed with their parent genes [10–12]. Expression of miRNAs in intergenic regions is controlled by the same mechanisms as protein coding genes, including upstream regulatory elements (i.e. promoters). Some regions of the genome encode clusters of miRNAs that can contain between two and dozens of miRNAs that are transcribed at the same time [13]. MiRNAs are usually transcribed by RNA polymerase II (but transcription via RNA polymerase III has also been described) as long primary miRNA precursors (pri-miRNAs) [14,15] that have extensive secondary structure containing a distinctive stem loop. In the nucleus, the pri-miRNAs are cleaved by the microprocessor complex which includes DGCR8 and the ribonuclease Drosha to generate approximately 70–100 nucleotide precursors (pre-miRNAs), which comprise only the stem-loop structure [16–18]. These precursors are translocated by Exportin-5 to the cytoplasm, where the loop is cleaved by the ribonuclease Dicer, to generate two single stranded mature miRNAs [19–25]. The miRNAs undergo strand selection [26] and one of the two miRNAs is often preferentially loaded into the RNA induced silencing complex (RISC) where it interacts with target mRNAs. Key members of the RISC are the argonaute proteins (Ago1–4), Dicer, TRBP and FMR1 [27–32]. Dicer and Ago2 appear to be essential for miRNA processing as the knockout mice are embryonic lethal [33–35].

MiRNAs can affect their targets by mediating mRNA decay or by inhibiting translation (reviewed in [36,37]). MiRNA interaction with target mRNAs can cause removal of the 3′ polyA tail and the 5′ cap, which causes destabilization and degradation of the mRNA [38–40]. When miRNAs interfere with translation without mRNA decay, they often prevent the initiation step of translation, but can also affect translation post-initiation [41,42].

A schematic showing the steps involved in miRNA biogenesis and the points at which steroid hormones regulate these steps is shown in Fig. 1 and described in Section 7.

Steroid hormone receptors (SHRs) are steroid-activated transcription factors that belong to the nuclear receptor superfamily (reviewed in [43]). Binding of ligand causes a conformational change that enables interaction with coregulators and binding to response elements to promote or repress gene expression. SHR response elements are often in the proximal promoters of genes, but can also be located at great distance from the target genes [44]. This review will focus on miRNAs regulated by SHRs as well as those that target SHRs directly and/or affect SHR gene regulation.

## 2. Estrogens and miRNAs

Many biological effects associated with estrogen receptor alpha (ERα) function can be linked to regulation of estrogen regulated miRNAs. In cells where estrogen is pro-proliferative, estrogen induced miRNAs tend to be known oncogenes (oncomirs). Conversely, estrogen repressed miRNAs tend to function as tumor suppressors. In the following section, we describe miRNAs induced or repressed by estradiol that participate in the diverse processes controlled by ERα or ERβ in different cell types. The complex interplay between miRNAs that regulate ERα and estrogen regulated miRNAs is depicted in Fig. 2. Estrogen regulated miRNAs, their known targets and associated biological effects are summarized in Table 1.

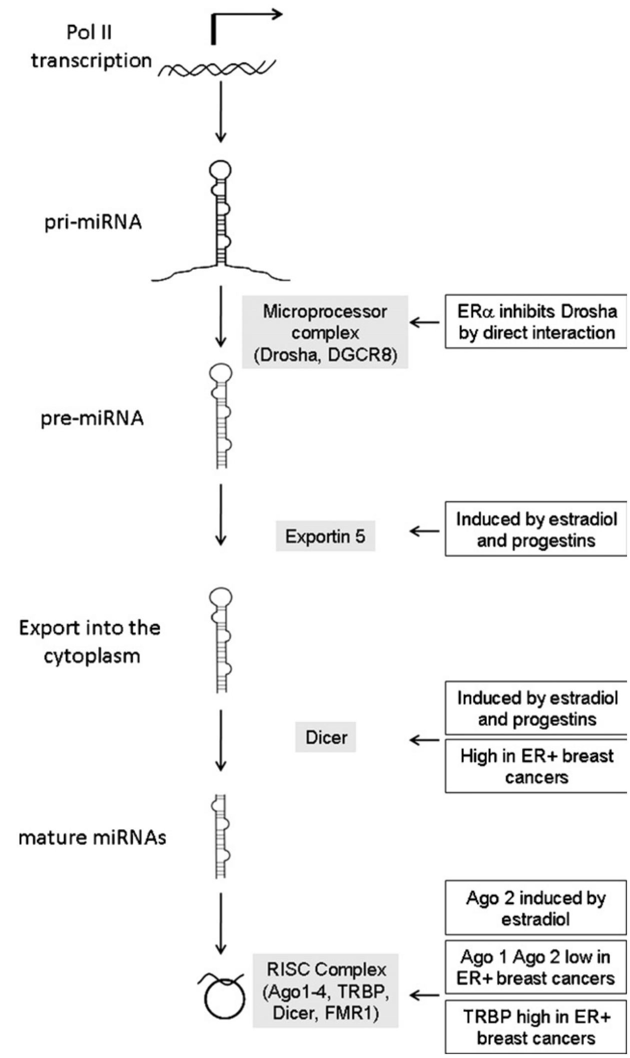


**Fig. 1.** MiRNA biogenesis and regulation by steroids. Pri-miRNAs, containing a hairpin loop, are generally transcribed by RNA polymerase II. Nuclease digestion of the pri-miRNA by the microprocessor complex generates the pre-miRNA. ERα inhibits Drosha through direct interaction [160]. The pre-miRNA is exported from the nucleus into the cytoplasm via Exportin 5, the expression of which is increased by estradiol and progestins [158]. Dicer cleaves the loop off of pre-miRNAs to generate mature miRNAs. Dicer expression is enhanced by estradiol and progestins and Dicer levels are higher in ERα positive versus negative breast cancers [54,56,156,157]. The mature miRNAs are loaded into the RISC where they can interact with target mRNAs. Ago2, a component of the RISC is induced by estradiol [159]. Ago1 and Ago2 are low in ERα+ breast cancers [156,159], while TRBP is high [156].

### 2.1. Estrogen regulated miRNAs

Several screens indicate that miRNAs are estrogen responsive [45–49]. In addition, miRNAs are differentially expressed between ERα positive and negative breast cancers [50–56]. Comparisons between mRNA and miRNA profiles reveal that estrogen regulated miRNA target clusters of estrogen responsive genes [57]. A study designed to characterize estrogen responsive miRNAs in zebrafish found that estrogen regulated miRNA expression is cell type specific, for example miR-196b which directly targets Hoxb8a [58] is increased with estradiol in the skin, but downregulated in the intestines and liver [46]. Estradiol can also regulate miRNA expression differently in normal and diseased tissue; for example, the expression of miR-26 is induced by estradiol in myometrial cells,

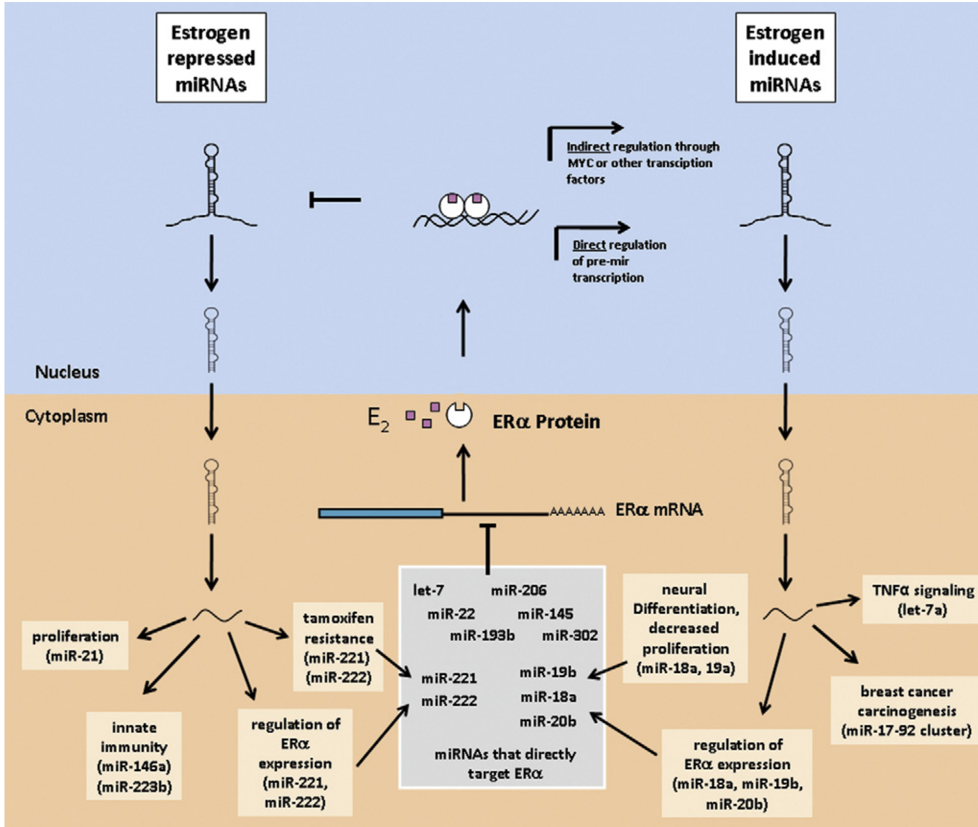


**Fig. 2.** MiRNAs regulated by ERα and miRNAs that regulate ERα. The 3′UTR of the ERα transcript is directly targeted by let-7, miR-206, miR-22, miR-145, miR-193b, miR-302, miR-221/222, miR-19b, miR-18a and miR-20b [55,76,80–86]. Estradiol (E2) bound ERα protein can regulate transcription of pre-miRNAs either by directly binding to the miRNA promoter (e.g., miR-21 [54]) or by indirectly modulating the expression of other transcription factors that bind to the promoter of the miRNAs (e.g., E2 upregulates c-myc, which then binds to the miR-17–92 promoter [55]). Estrogen induced miRNAs are involved in breast carcinogenesis [66–68], TNFα signaling [64], neural differentiation [69] and regulation of ERα expression [55,70]. Estrogen repressed miRNAs are involved in proliferation [74], tamoxifen resistance [76,77], innate immunity [79] and regulation of ERα expression [75,76].

but suppressed in leiomyoma cells [59]. Below we discuss some of the functional implications of estrogen induced and repressed miRNAs identified to date.

### 2.2. Estrogen induced miRNAs

The let-7 family of miRNAs is induced by estradiol [54], and tends to be lower in breast cancers compared to normal breast [60]. Like let-7, the miR-200 family is often associated with an ERα+, epithelial phenotype [61–63] and is also induced by estradiol [54]. Let-7 is also involved in estrogen-mediated regulation of innate immune function. One of the mechanisms through which ERα affects lipopolysaccharide (LPS) induced TNFα signaling is through upregulation of let-7a, which directly targets kappaB-Ras2 by binding to its 3′UTR [64].

Some miRNAs reside in clusters where the pri-mirs are expressed on one transcript [65]. The miR-17–92 cluster and its paralogs are often overexpressed during breast carcinogenesis and the expression of the miRNAs encoded in these clusters is increased with estradiol treatment [66–68]. This occurs indirectly when as estradiol stimulates c-myc, which binds to the promoter of the miR-17–92 cluster [55]. In neuroblastoma, estrogen decreases pro-

**Table 1**
Estrogen regulated miRNAs. Summary of miRNAs modulated by estrogen, their known targets and biological effects.

| MiRNA | Regulation | Biological effects | Targets | Ref. |
|---|---|---|---|---|
| Let-7 family | Upregulated by estradiol high in ER+ breast cancers | Promotes an epithelial phenotype innate immunity | kappaB-Ras2 | [54,60,64] |
| MiR-17–92 cluster and paralog (miR-18a, miR-19) | Upregulated by estradiol high in breast cancers | Decreased proliferation and increased differentiation in neural cells | ERα | [55,66–68,70] |
| MiR-21 | Directly repressed by ER high in ER-breast cancers | Promotes proliferation inhibits cell death | Pdcd4, PTEN, Bcl-2 | [74] |
| MiR-26a | Upregulated by estradiol in myometrial cells downregulated by estradiol in leiomyoma cells | Regulation of PGR expression | PGR | [47,59] |
| MiR-146a | Repressed by estradiol in splenocytes and breast cancer cells | Innate immunity | | [79] |
| MiR-181a | Repressed by estradiol in breast cancer cells | Regulation of PGR expression | PGR | [47] |
| MiR-196b | Up with estradiol in zebrafish skin down with estradiol in zebrafish intestines and liver | | Hoxb8a | [58] |
| MiR-200 family | Upregulated by estradiol high in ER+ breast cancers | Promotes an epithelial phenotype | | [54,61–63] |
| MiR-221/222 | Directly repressed by ER high in breast cancers | Tamoxifen resistance | ERα, p27kip, FOXO3, BIM | [75–77] |
| MiR-223 | Repressed by estradiol in splenocytes | Innate immunity | | [79] |

liferation and induces neuronal differentiation [69]. However, in MYCN-driven tumors, induction of MYCN-induced miRNAs (miR-18a and miR-19a) that directly target and repress ERα expression, may lead to aberrant regulation of ERα in primitive sympathetic cells and interfere with normal neuroblast differentiation, thereby representing a potential tumorigenic mechanism in the etiology of neuroblastoma [70].

### 2.3. Estrogen repressed miRNAs

MiR-21, a well studied oncomir that promotes cell proliferation and inhibits cell death [71–73], is repressed by estradiol and this effect is blocked by ERα antagonists [74]. ERα directly suppresses pre-mir-21 transcription by binding to its promoter region [54]. Treatment with estradiol causes an increase in the miR-21 target genes Pdcd4, PTEN and Bcl-2 [74].

MiR-221 and miR-222 share the same seed sequence and are located in close proximity on the X chromosome [65]. These miRNAs are repressed by ERα [75] and are often overexpressed in ERα− breast cancers [76]. MiR-221/222 are expressed at high levels in tamoxifen resistant breast cancers [77] and appear to play a role in tamoxifen resistance through at least two mechanisms. Since they directly target ERα, overexpression of miR-221/222 is sufficient to render ERα+ cells resistant to tamoxifen [76]. MiR-221/222 also directly target the cell cycle inhibitor p27kip, contributing to their ability to induce tamoxifen resistance [77].

In addition to affecting innate immunity by inducing let-7, estradiol also influences innate immunity by repressing miR-146a and miR-223b. In splenocytes, estradiol suppresses TNFα and induces IFNγ and iNOS [78], and these effects may be mediated by repression of miR-146a and miR-223b [79]. Both of these miRNAs repress LPS induced IFNγ, while only miR-146a represses iNOS [79].

### 2.4. MiRNAs that target ERα

Several miRNAs directly target the ERα transcript, including miR-206, miR-221, miR-222, miR-22, let-7, miR-18a, miR-19b, miR-20b and miR-145 [55,76,80–83]. Most miRNAs that target ERα are highly expressed in ERα− breast cancers and low in ERα+ breast cancers. Overexpression of these miRNAs causes a decrease in ERα protein, ERα signaling and suppression of ERα target gene expression. Although most of these miRNAs bind to the 3′UTR of the ERα transcript, miR-145 affects the translation of ERα by binding to a site in the ERα coding region [83].

Since estrogen signaling is pro-proliferative in breast cells, miRNA-mediated ER repression generally causes a decrease in proliferation [84]. While miR-206 and miR-221/221 directly target ERα and low in ERα+ cells [76,80], they appear to have opposing effects on breast cancer cell proliferation. Mir-106 directly represses MET, while increasing FOXO3 and BIM, resulting in decreased proliferation. Conversely, miR-221 and miR-222 increase proliferation by directly targeting and decreasing FOXO3 and BIM [75]. Clearly further work is necessary to fully understand which miRNAs are the dominant regulators of ERα expression and activity in breast cancer.

While most screens have looked for correlations between ERα and miRNA expression levels, Leivonen et al. performed a high throughput, functional screen to identify miRNAs that potentially target ERα. In this screen, a library of miRNA mimics was transfected into ERα+ MCF7 cells and cell lysates probed for alterations in ERα protein levels [85]. They found 21 miRNAs that can decrease ERα protein and suppress growth, the most potent being miR-18a, miR-18b, miR-193b, miR-206 and miR-302 [85].

In some cases, the interplay between miRNA and ERα is in the form of a negative feedback loop. MiR-206 directly targets the 3′UTR of ERα and estradiol represses miR-206; however, this may be an indirect effect [86]. In a similar negative feedback loop, the ERα 3′UTR is directly targeted by miR-221/222, while ERα binds directly to the promoter region of the pre-mir-221/222 gene and recruits NCoR and SMRT to repress expression of miR-221/222 [75]. Lastly, estradiol induces the miR-17–92 cluster and its paralogous clusters. Three of the members of these paralogs, miR-18a, miR-19b and miR-20b, target ERα directly [55].

Since miRNAs rely on complementary base pairing, SNPs in their target sites can affect the efficacy of binding and activity. The human ERα 3′UTR contains a SNP associated with an increased risk for breast cancer in premenopausal women, but does not correlate with increased risk in postmenopausal women [87]. While the functional assays remain to be done, the SNP lies within the miR-453 binding site in the ERα 3′UTR and is predicted to decrease the binding affinity of the miRNA. The authors predict that premenopausal women (with high circulating estradiol) who have this SNP would have an increased risk of breast cancer [87]. Interestingly, in rodents a SNP occurs in the ERα 3′UTR in the miR-206 binding site which increases its binding affinity and activity resulting in decreased ERα [86]. Since the ERα 3′UTR is over 4 kb in length and contains multiple miRNA binding sites, it is possible that more SNPs will be identified that affect miRNA binding.

The repression of ERα activity can be indirect, as is the case with miR-27a. ERα can induce gene transcription at non-consensus estrogen response elements (EREs) or ERE half sites through specificity protein (Sp) transcription factors [88]. MiR-27a affects ERα signaling indirectly by repressing the zinc finger protein ZBTB10, a repressor of Sp1 and Sp4 [89]. The ERα promoter has Sp binding sites, to which the Sp proteins bind and repress ERα expression [90]. Consequently the overall effect of miR-27a expression is decreased ERα protein expression and activity [90].

Little is known regarding ERβ and miRNAs; however, one study shows that ERβ is targeted by the estradiol-induced miRNA, miR-92, in breast cancer cells [91].

## 3. Androgens and miRNAs

Androgens play a role in development, differentiation and function of the prostate [92,93]. In the prostate, androgens serve to promote cell growth and prevent apoptosis [94–97] and while this can be achieved by directly regulating gene expression at the promoter level, androgen regulated miRNAs also play a role. While initially dependent on androgens for growth, prostate cancers often progress to androgen independence. However, androgen receptor (AR) signaling remains high in androgen independent (AI) prostate cancer. MiRNAs are now known to be involved in prostate cancer initiation and progression to androgen independence. Androgen regulated miRNAs, their known targets and resulting biological effects are summarized in Table 2.

### 3.1. Androgen regulated miRNAs

Profiling of prostate tumors reveals that miRNAs that are highly expressed in AI prostate cancers (in which androgen signaling is high) correspond to low expression of their target mRNAs [98–100]. In the female mouse liver, treatment with testosterone causes an increase in 6 miRNAs [101]. Only one of those miRNAs, miR-122, has a putative androgen response element (ARE) in its promoter region [101]. In this system, testosterone regulated miRNAs have an impact on the overall steroid biology, as testosterone induced miR-22 with a concomitant decrease in two of its target genes, ERα and aromatase (CYP19A1) [101].

As has been previously discussed, the miR-221 cluster is repressed by ERα binding to an estrogen response element in its promoter. The miR-221 cluster also has a putative ARE in its

**Table 2**
Androgen regulated miRNAs. Summary of miRNAs modulated by androgens, their known targets and biological effects.

| MiRNA | Regulation | Biological effects | Targets | Ref. |
|---|---|---|---|---|
| MiR-21 | Directly upregulated by AR high in AR+ cells | Promotes AI growth and migration, inhibits apoptosis | MARCKS | [108,109] |
| MiR-22 | Upregulated by testosterone in mouse liver | Influences steroid biology by decreasing ERα and aromatase | ERα, CYP19A1 | [101] |
| MiR-101 | Upregulated by androgens high in prostate cancer cells | Epigenetic gene regulation through histone modification | Ezh2 | [115] |
| MiR-122 | Upregulated by testosterone in mouse liver | | | [101] |
| MiR-125b | Directly upregulated by AR high in prostate cancer cells | Inhibition of apoptosis | Bak1 | [113] |
| MiR-146a/b | Low in AI cell lines | Inhibition of proliferation, migration and invasion | ROCK | [107] |
| MiR-221/222 | Repressed by androgens high in AI prostate cancer | Increased proliferation | p27/kip1 | [99,102–106] |

promoter and is found to be repressed by androgens in androgen responsive prostate cell lines [99]. These miRNAs are often overexpressed in cancers, including AI prostate cancer [102–104]. MiR-221/222 are high in AI prostate cancer and promote androgen independent growth [104,105]. As was observed in breast cancer cells, one of the mechanisms through which increased miR-221/222 mediates proliferation is through targeting of p27/kip1 [104,106].

Profiling of androgen dependent and independent cell lines found that miR-146a and miR-146b are low in the AI cell lines. Since miR-146a serves to inhibit proliferation, migration and invasion by targeting ROCK, a kinase required for hyaluronan-mediated tumorigenesis, its loss in AI cells contributes to the aggressive nature of these cells [107].

The oncomir miR-21 is directly upregulated by AR binding to its promoter [108]. MiR-21 is expressed at lower levels in AR− compared to AR+ prostate cancer cells [109] and overexpression of miR-21 is sufficient to make androgen dependent cells become androgen independent [108]. MiR-21 promotes proliferation and migration and inhibits apoptosis. Inhibition of miR-21 causes a decrease in many steps involved in metastasis including motility, invasion, intravasation and colonization at distant sites [110–112]. One of the mechanisms through which miR-21 affects cell motility in prostate cells is by direct targeting of MARCKS, a regulator of cytoskeletal structure [109].

AR binds directly to the miR-125b promoter and induces its expression. MiR-125b is high in AI cell lines and clinical prostate cancers with high Gleason grades. This oncomir promotes growth by directly targeting the pro-apoptotic gene Bak1, thereby allowing evasion of apoptosis [113]. In prostate cells, miR-34 expression is induced by DNA damaging agents in an AR-dependent manner and this miRNA is required for p53 dependant apoptosis [114].

In addition to affecting direct targets, some miRNAs can have a more global impact on gene expression by affecting epigenetic gene regulation. An example of this is the androgen upregulated miRNA, miR-101, which directly targets Ezh2, a histone methyltransferase. Overexpression of miR-101 in prostate cells results in a reduction of Ezh2, histone methylation and invasive capacity [115].

### 3.2. MiRNAs that regulate AR transcriptional activity

While there are no reports to date on miRNAs that directly target AR itself miRNAs can affect AR signaling without altering AR levels. MiR-331-3p directly targets ERBB2 and is expressed at low levels in prostate tumors that overexpress ERBB2. Introduction of miR-331-3p into cells results in the loss of ERBB2 expression, which dampens AKT phosphorylation and AR phosphorylation [116]. Since phosphorylation of AR promotes its transcriptional activity [117,118], there is an inhibition of AR mediated signaling without affecting AR levels in miR-331-3p transfected cells [116]. While the mechanism of action remains to be elucidated, miR-221/222 can also interfere with AR regulated gene expression without affecting AR itself [104].

## 4. Progesterone and miRNAs

The biological roles of the natural ligand progesterone and progesterone receptors (PGRs) are complex. In the breast, progesterone is both proliferative and differentiative, with PGRs being required for both expansion and differentiation of lobular alveoli during pregnancy. Recently several groups have shown that progesterone and progestins mediate the expansion of a stem cell population in normal human breast [119], normal mouse mammary gland [120,121] and breast cancer [122]. Since miRNAs influence stem cells (see recent studies [123–125] and reviews [126,127]), it is interesting to speculate that progesterone regulated miRNA could mediate the effects of progesterone on stem cells in the breast. However, to date, direct effects of progesterone on miRNA expression have not been investigated in normal or malignant breast cells. In contrast, progesterone regulation of miRNAs has been examined quite intensively in the uterus, another organ exquisitely sensitive to the female sex steroids.

### 4.1. Progesterone regulated miRNAs

The uterus is a complex organ in which steroid hormones influence both the epithelial and stromal compartments of the endometrium as well as the smooth muscle cells of the myometrium. In the endometrium, it is clear that progesterone counteracts estrogen-mediated proliferation. To determine whether miRNAs play a physiological role in modulating hormonal control of gene expression in the endometrium, the Pollard laboratory examined differential expression of miRNAs in epithelial cells isolated from endometrial biopsies in the late proliferative versus mid-secretory phases [128]. Twelve miRNAs significantly upregulated in the mid-secretory phase are predicted to target many cell cycle genes, consistent with suppression of cell proliferation in the endometrial epithelium during the secretory phase when progesterone levels peak [128].

Even more direct evidence for progestin-mediated regulation of miRNAs comes from studies treating PGR positive cells with a synthetic progestin, medroxyprogesterone acetate (MPA). In the first study, progestin regulated miRNAs were identified by treating a spontaneously transformed leiomyoma cell line (T-LSMC) and a leiomyosarcoma cell line (SK-LMS-1) with MPA, without or with the synthetic antagonist RU-486 [59]. Many of the identified miRNAs were also found to be differentially expressed in paired normal myometrium versus leiomyoma (uterine fibroids), and may play a role in the pathogenesis of these common benign, yet troublesome, tumors. In another study, paired eutopic and ectopic endometrium and isolated endometrial cells were profiled for differentially expressed miRNAs in order to identify those associated with endometriosis [129]. In the original and subsequent study by this group [130] endometrial epithelial and stromal cells were treated with MPA and some of the endometriosis-associated miRNAs were found to be regulated by progestin, with effects abrogated by RU-486. This work and the general implications of hormonally regulated miRNA in the endometrium in normal and disease states are summarized by the authors [59].

MiR-320 [131] and let-7 [132] are regulated by progesterone during embryo implantation in the rat. Other miRNAs are differentially expressed in the mouse uterus between implantation sites and inter-implantation sites [133]. Compared with inter-implantation sites, 13 miRNAs are upregulated (including many members of the let-7 family, miR-143, miR-298, miR-21, miR-20a and miR-26a) while two (miR-290-5p and miR-292-5p) are downregulated at least 2-fold at implantation sites in the mouse uterus [133]. Perhaps the most profound effect on uterine physiology to date is the finding that P4/PGR affects uterine contractility during labor via regulation of ZEB1 and the miR-200 family [134].

### 4.2. MiRNAs that target PGR

The PGR 3′UTR is unusually long (>13 kb in humans), containing an abundance of predicted miRNA target sites; however, few studies have examined miRNA regulation of PGR. Two miRNAs inhibited by estradiol (miR-26a and miR-181a) directly target the PGR 3′UTR and reduce PGR mRNA and protein [47]. Thus, estradiol-mediated downregulation of these miRNAs allows for increased expression of PGR, implying that estradiol-mediated upregulation of PGR in MCF7 cells has two aspects. The first is direct binding of ER to the PGR promoter, and the second is an indirect posttranscriptional effect via downregulation of miRNAs that target PGR. In summary, although miRNAs that correlate with PGR status in breast cancer have been identified [53], to date miR-26a and miR-181a are the only miRNAs demonstrated to directly target PGR.

## 5. Glucocorticoids, mineralocorticoids and miRNAs

Glucocorticoids (GCs) are stress-induced steroid hormones produced in the adrenal cortex. They are known to suppress cell growth and proliferation processes in the brain and contribute to cognition, memory, and emotion [135,136]. GCs are widely used as immunosuppressant drugs as they inhibit immune cell proliferation through induction of apoptosis in T lymphocytes [137]. In humans, the most important GCs are cortisol (hydrocortisone) and cortisone. GCs activate two types of nuclear receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). GR controls a variety of physiological functions; such as, metabolism, development, and reproduction; whereas MR is critical for controlling sodium and potassium transports in epithelial cells and plays important roles in the pathophysiology of hypertension and cardiac fibrosis. In addition to glucocorticoids, MR can also bind mineralocorticoids, aldosterone and progesterone [138].

### 5.1. Glucocorticoid and mineralocorticoid regulated miRNAs

Activation of GR and MR by glucocorticoids has pronounced effects on metabolism, differentiation, proliferation and cell survival in many tissues. Given the known role of miRNAs in regulating many of these processes, it is logical to hypothesize that specific miRNAs are also induced or repressed by GC signaling. Furthermore, transcriptional activation of GR and MR is likely to induce the expression of intronic miRNAs transcribed concomitant with GR-responsive genes. To test this hypothesis, Rainer et al. correlated miRNA levels with expression data of their host genes in cell lines and clinical samples of children with acute lymphoblastic leukemia (ALL) undergoing systemic GC monotherapy [139]. At least five miRNAs were significantly regulated by GC therapy and a fraction of mature miRNAs regulated by GC could be inferred from expression data of their host genes [139]. Importantly, the miR-15/16 cluster, which induces cell cycle arrest, is upregulated by GC in a subset of ALL patients and cell lines, consistent with the known apoptotic effect of GCs in immature lymphoblasts. Overexpression of miR-15b/16 mimics increased GC sensitivity in leukemia cell lines and silencing miR-15b/16 with inhibitors decreased GC sensitivity *in vitro* [139], further suggesting that miRNA regulation is a vital component of GC signaling.

### 5.2. MiRNAs that regulate GR and MR transcriptional activity

The transcriptional activity of GR and MR depends on multiple factors [140,141], including protein variants produced by alternate splicing, the use of different promoters, alternate translation initiation sites, coactivators, corepressors and post-translational modifications such as phosphorylation, ubiquitinylation, sumoylation and acetylation (for a review for each receptor, see [140,142]). However, there is evidence that absolute GR and MR protein levels also influence glucocorticoid-responsiveness in a given cell [143], suggesting that miRNA-mediated downregulation of GR and MR could affect their transcriptional activity [136]. MiR-18 and miR-124a decrease GR protein levels and GR-mediated transactivation in neuronal cells [144]. Overexpression of both miRNAs attenuates GR-mediated transactivation and reduces the induction of a GR-response gene GILZ; however, only miR-124a is able to directly bind the GR 3′UTR. Neuronal differentiation of P19 (embryonal carcinoma) cells is associated with strong miR-124a upregulation and resultant downregulation of GR protein, and the expression level of miR-124a in brain tissues is high enough to effectively reduce GR [144], further confirming the role of these miRNAs in brain GR regulation. MiR-18 is also implicated in the regulation of the GR 3′UTR *in vitro*, and increased miR-18 may, in part, be responsible for the aberrant GR signaling and increased susceptibility towards stress in Fischer 344 rats [145]. Interestingly, the 3′UTR of the human MR (also known as *NR3C2*) also appears to be targeted by miR-124, as well as miR-135a [146]. However, a functional link between miR-124 or miR-135 and the physiological role of MR remains to be elucidated.

MiRNAs can modulate the sensitivity to corticosteroid therapy without directly affecting GR or MR expression [147], suggesting that they act on other effectors of the response to GC. Kotani et al. demonstrate that expression of miR-128b and miR-221 in ALL results in increased steroid-induced apoptosis, and they suggest that miR-221 downregulation of CDKN1B (encoding p27) mediates this effect [147].

## 6. MiRNAs that affect SHR coregulators

MiRNAs can target co-regulators of SHR transcriptional activity and may thereby contribute to the cell, tissue, and gene specific activity of SHRs. PGC-1α, a coregulator of MR [140], is regulated by miR-696 in mouse muscle cells [148]. Some miRNAs, such as miR-20b and miR-206, directly target ERα [55,80] and also modulate SHR coregulator expression. MiR-20b reduces the levels of the coactivator AIB1 [55], whereas miR-206 affects estrogen signaling by targeting the coregulators SRC-1, SRC-3 and GATA-3. Consequently, estrogen signaling is disrupted by miR-206 even in the presence of exogenous ERα that cannot be targeted by miR-206 [149]. MiR-17-5p directly represses AIB1 through two sites, one of which is in the coding region. Overexpression of miR-17-5p suppresses ERα transcriptional activity and also inhibits estrogen dependent proliferation and anchorage independent growth [150]. MiRNAs that target both SHRs and their coregulators may provide a “failsafe” mechanism by which the ability of the SHR to serve as a functional transcription factor is repressed.

In addition to miRNAs affecting transcriptional coregulators, they may target other proteins involved in different aspects of SHR activity. For instance, it is possible that expression of proteins involved in cytoplasmic rapid signaling, nuclear transport or chaperones may be regulated by miRNAs.

## 7. Steroid regulation of miRNA machinery

MiRNAs are essential for development and differentiation of all tissues including those heavily influenced by steroid hormones such as the mammary gland and uterus. Indeed, Dicer is required for female reproductive tract development and fertility in the mouse [151–153] (and reviewed by [154]).

Several studies have implicated ERα and estrogen signaling in regulation of the miRNA machinery. In ovarian cancers, low ERα is correlated with low Dicer levels and a resultant global decrease in miRNA levels [155]. Expression of Dicer is lower in breast cancer cell lines and clinical samples that have undergone epithelial to mesenchymal transition and lost expression of ERα [156,157]. MiR-222 and miR-29a, which are overexpressed in ERα− breast cancers directly target Dicer, which may explain why Dicer is lower in ERα− breast cancers [56]. In the mouse uterus, Exportin 5, which transports pre-miRNAs into the cytoplasm from the nucleus, is increased with estradiol as well as progesterone [158]. Interestingly, miRNAs were found to be more effective at reducing their targets in ERα− compared to ERα+ breast cancer cells, which may be due to dysregulation of components of the RISC that alter their efficacy [156]. Ago1 and Ago2 are high in ERα− breast cancers [156,159], while Dicer and TRBP are low [156]. Dicer itself is induced by estradiol [54] as well as by progestins [158]. Paradoxically, Ago2 is upregulated by estradiol even though it is high in ERα− cells [159].

Yamagata et al. found that ERα interacts directly with the components of the Drosha microprocessor complex and interferes with their ability to generate pre-miRNAs from pri-miRNAs. As a result, estradiol treatment causes a decrease in the pre-miRNA and the mature miRNA, but not the pri-miRNA [160].

Bioinformatic analysis of ERα+ and ERα− breast cancers shows that transcripts higher in ERα+ tumors have longer 3′UTRs and are enriched for miRNA binding site motifs compared to transcripts higher in ERα− cells [161]. It is likely that reduced Dicer expression is related to the global downregulation of the miRNAome observed in cancer and it is thought that the reduced number and abundance of miRNAs in human cancers reflects an altered differentiation state [162,163]. Three separate studies of ERα positive versus negative breast cancers found that the majority of differentially expressed miRNAs are less abundant in ERα− tumors [50,51,164].

## 8. Conclusions and significance

Traditionally, SHRs have been thought to exert their effects at the 5′ regulatory regions. However, in the future, it is likely that we will think of steroid responsive genes as being controlled by SHR not only at the 5′UTR, but also at the 3′UTR by steroid responsive miRNAs. Perhaps the ultimate reporter for a SHR-regulated gene will have both the relevant promoter cloned upstream of luciferase and also the 3′UTR of the same gene cloned downstream of luciferase. Conventionally, a steroid hormone responsive gene was considered to be directly regulated by an SHR if inhibitors of translation, such as cycloheximide, did not affect their regulation. However, since miRNAs are not translated, it is possible that such a gene could actually be exclusively or partially controlled by a SHR regulated miRNA(s). In other words, it is conceivable that a gene(s) could be not only upregulated by SHR binding to the promoter, but also controlled by SHR-mediated downregulation of a miRNA(s), which would relieve repression by permitting translation. In this way SHRs could exert post-transcriptional control of many genes when a steroid hormone cue triggers downregulation of miRNAs, relieving the repression of a whole set of genes. In effect the SHR regulation of miRNAs would be a way to amplify the response. One physiologically relevant situation in which such a mechanism might be utilized is secretory activation in the mammary gland whereby translation of many milk protein and lipid synthesis genes is dramatically increased in response to a precipitous drop in progesterone. Indeed, a dramatic downregulation of many miRNAs occurs between pregnancy and lactation [165].

In conclusion, researchers that study SHRs in the context of endocrinology, physiology, developmental biology and hormone-related malignancies are swiftly recognizing the myriad ways in which miRNAs can impact SHR action. Many future functional studies will reveal the full impact that these small non-coding RNAs have on SHR biology.

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# MicroRNAs Link Estrogen Receptor Alpha Status and Dicer Levels in Breast Cancer

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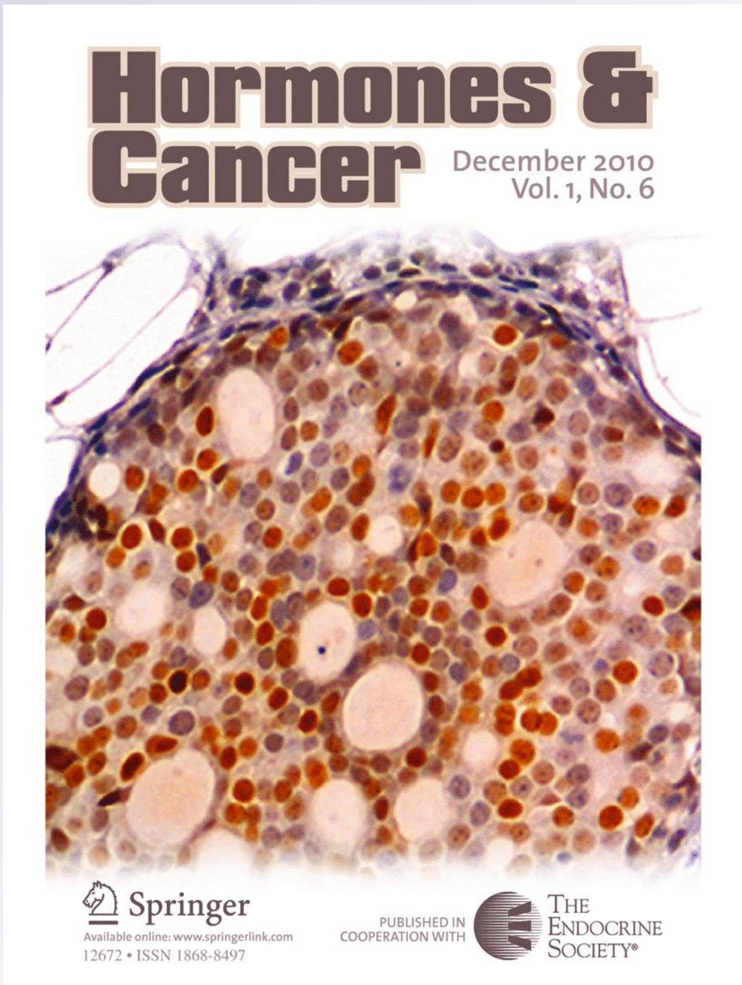

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# MicroRNAs Link Estrogen Receptor Alpha Status and Dicer Levels in Breast Cancer

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**Abstract** To identify microRNAs (miRNAs) associated with estrogen receptor (ESR1) status, we profiled luminal A, ESR1+ breast cancer cell lines versus triple negative (TN), which lack ERα, progesterone receptor and Her2/neu. Although two thirds of the differentially expressed miRNAs are higher in ESR1+ breast cancer cells, some miRNAs, such as miR-222/221 and miR-29a, are dramatically higher in ESR1− cells (~100- and 16-fold higher, respectively). MiR-222/221 (which target ESR1 itself) and miR-29a are predicted to target the 3′ UTR of *Dicer1*. Addition of these miRNAs to ESR1+ cells reduces Dicer protein, whereas antagonizing miR-222 in ESR1− cells increases Dicer protein. We demonstrate via luciferase reporter assays that these miRNAs directly target the *Dicer1* 3′ UTR. In contrast, miR-200c, which promotes an epithelial phenotype, is 58-fold higher in the more well-differentiated ERα+ cells, and restoration of miR-200c to ERα− cells causes increased Dicer protein, resulting in increased levels of other mature miRNAs typically low in ESR1− cells. Together, our findings explain why Dicer is low in ERα negative breast cancers, since such cells express high miR-221/222 and miR-29a levels (which repress Dicer) and low miR-200c (which positively affect Dicer levels). Furthermore, we find that miR-7, which is more abundant in ERα+ cells and is estrogen regulated, targets growth factor receptors and signaling intermediates such as EGFR, IGF1R, and IRS-2. In summary, miRNAs differentially expressed in ERα+ versus ERα− breast cancers actively control some of the most distinguishing characteristics of the luminal A and TN subtypes, such as ERα itself, Dicer, and growth factor receptor levels.

**Keywords** Dicer · miRNA · ESR1 · Epithelial to mesenchymal transition · Breast cancer

**Abbreviations**

| | |
|---|---|
| EMT | Epithelial to mesenchymal transition |
| ESR1 | Estrogen receptor alpha gene |
| ERα | Estrogen receptor alpha protein |
| miRNA | MicroRNA |
| RISC | RNA induced silencing complex |
| TRBP | Tar RNA-binding protein |
| TN | Triple negative |
| UTR | Untranslated region |

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## Introduction

Since specific microRNAs (miRNAs) are capable of regulating hundreds of mRNAs simultaneously, it was not unexpected to find that miRNA profiling can distinguish breast cancer subtypes [5]. MiRNAs function by binding to the 3′ untranslated region (UTR) of their targets and either prevent translation or cause mRNA degradation. The human RNase III-type nuclease Dicer performs the final step of biogenesis of miRNAs in which the pre-miRNA stem loop is cleaved to produce a mature miRNA. The mature miRNA is then incorporated into the RNA induced silencing complex, consisting of Dicer, Tar RNA-binding protein (TRBP), argonaute proteins, and several other proteins, which guide the mature miRNA to specific target mRNAs. Conditional deletion of Dicer enhances transformation and tumorigenesis, and Dicer functions as a haploinsufficient tumor suppressor [36, 37]. Three separate studies of estrogen receptor alpha (ESR1) positive (ESR1+) versus negative (ESR1−) breast cancers found that the majority of differentially expressed miRNAs are less abundant in ESR1− tumors [5, 26, 45]. It is likely that reduced Dicer expression is related to the global down-regulation of the miRNAome observed in cancer, and it is thought that the reduced number and abundance of miRNAs in human cancers reflects an altered differentiation state [7, 43]. Expression of Dicer is lower in breast cancer cell lines and clinical samples that have undergone epithelial to mesenchymal transition (EMT) [23], and Dicer is differentially expressed between ESR1 positive versus negative breast tumors [11]. Similarly, lower Dicer levels are associated with loss of ESR1 in ovarian cancers [18].

Dicer levels are regulated by let-7 via binding sites in the *Dicer1* 3′ UTR and coding region [19, 60]. MiR-103/107 was recently reported to repress *Dicer1* through three sites in the Dicer 3′ UTR [44]. We observed that the 3′ UTR of *Dicer1* also contains well-conserved binding sites for miR-221/222, which directly target ESR1 [16, 65] and for miR-29a. We find these to be the most differentially expressed miRNAs higher in ERα- negative versus ERα+ breast cancer cells. We hypothesized that miR-221/222 directly represses not only ESR1, but also Dicer itself, and that miR-29a also directly targets Dicer, possibly explaining why Dicer is lower in ERα negative breast cancers.

In contrast to miR-221/222 and miR-29a, the majority of differentially expressed miRNAs are higher in ERα+ cells, and of these, miR-200c is the most differentially expressed. We previously observed that restoration of miR-200c to dedifferentiated endometrial cancer cells increased *Dicer1* mRNA levels [14]. We now demonstrate that restoration of miR-200c to triple negative (TN) breast cancer cells (that lack ERα, progesterone receptors, and Her2neu expression) causes an increase in Dicer protein resulting in an increase in the mature form of some of the miRNAs that are typically lower ERα− cells.

## Materials and Methods

### Cell Culture and Hormone Treatments

MCF7 and T47D breast cancer cells, which belong to the luminal A subtype, were grown in DMEM, L-glutamine, penicillin/streptomycin, and fetal bovine serum (FBS). MDA-MB-231 breast cancer cells (triple negative subtype) were grown in MEM containing FBS, HEPES, NEAA, L-glutamine, penicillin/streptomycin, and insulin. BT549 breast cancer cells (triple negative subtype) were grown in RPMI containing FBS and insulin. Hec50 cells were grown in DMEM containing FBS and penicillin/streptomycin. Cells were maintained at 37°C and 5% $CO_2$. The identity of all cell lines was confirmed using the Identifiler DNA profiling kit (ABI) in the University of Colorado Cancer Center Sequencing Core Facility.

MCF7 cells were grown in phenol red-free media containing charcoal stripped serum for 24 h prior to hormone treatments. The cells were treated with ethanol, 10 nM estradiol, or a combination of 10 nM estradiol and 1 μM ICI 182,780 (ICI, Tocris Bioscience) for 24 h before harvesting total RNA using Trizol (Invitrogen), which retains both small RNA species such as miRNAs and larger RNAs such as mRNAs and rRNAs.

### Immunoblotting

Whole cell lysates made with RIPA buffer were separated on SDS PAGE gels and transferred to PVDF membranes, blocked, and probed overnight at 4°C. Primary antibodies used were: ERα (clone AER611,NeoMarkers), E-cadherin (clone NCH-38, DAKO), ZEB1 (rabbit polyclonal, Dr. Doug Darling, University of Louisville); N-cadherin (clone 13A9, Upstate), Vimentin (clone V9, Sigma), Dicer (rabbit polyclonal, Sigma), α-tubulin (clone B-5-1-2, Sigma), EGFR (rabbit polyclonal, Cell Signaling Technology), IGF1Rβ (rabbit polyclonal (C-20), Santa Cruz Biotechnology), IRS-1 [56], IRS-2 (rabbit polyclonal (H-205), Santa Cruz Biotechnology), ERK1/2 (MAPK), phospho-specific and total (rabbit polyclonal, Cell Signaling Technology). After incubation with HRP-conjugated secondary antibodies, results were detected using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer).

### MiRNA Microarray Profiling

Total RNA was prepared using Trizol (Invitrogen). Labeling, hybridization to miRNA microarray slides, and feature extraction was performed by ThermoFisher using the Agilent miRNA microarray platform containing all miRNAs in the Sanger version 10 database. Each miRNA probe is spotted in seven locations to allow for statistical analysis to be performed. Relative intensity data for the multiple probes for each miRNA was subjected to statistical filtering. Probes with $p$ values ≤0.05 in at least two of the eight slides were retained for further analysis. For the luminal versus triple negative screen, the filtered array data was analyzed and clustering was performed using GeneSpring GX 10 (Agilent

Technologies). Data was filtered using a twofold change cutoff and a $p$ value of 0.05 (ANOVA, Benjamini Hochberg FDR multiple testing correction). For the graphical representation of the data, averages were taken for T47D and MCF7 to generate the ERα+ values and averages for MDA-MB-231 and BT549 were used to generate the ERα− values.

## Real Time RT-PCR

TaqMan MiRNA Reverse Transcription kit was used to generate cDNA from total RNA using a miR-7, miR-29a, miR-221, miR-22, miR-193, miR-148a, or U6 specific primers (Applied Biosystems). For normalization, real time RT-PCR was performed on the cDNA using 18S rRNA primers and probe (Applied Biosystems). For miR-34a, miR-19b, miR-20a, and miR-106a, poly A tailing and reverse transcription was performed using the NCode miRNA qRT-PCR kit (Invitrogen). SYBR green real time RT-PCR was performed using the Universal Forward Primer (Invitrogen) and a miRNA specific primer. For normalization, levels of β-actin were quantified using gene-specific primers. The relative miRNA levels were calculated using the comparative Ct method (ΔΔCt). Briefly, the Ct (cycle threshold) values for the rRNA, U6, or actin were subtracted from Ct values of the miRNA to achieve the ΔCt value. The $2^{-\Delta Ct}$ was calculated and then divided by a control sample to achieve the relative miRNA levels (ΔΔCt). Reported values are the means and standard errors of three biological replicates.

## Immunohistochemistry

Sections were cut at 4 μm and heat immobilized. After deparaffinization and antigen retrieval, endogenous peroxidase was blocked. Sections were incubated with primary antibody for 1 h. Primary antibody used was Estrogen Receptor alpha (clone 1D5, Dako). Vectastain Elite ABC kit (Vector Labs) was used for serum blocking and antibody detection, followed by incubation with 3, 3′-diaminobenzidine (Dako) for protein visualization.

## In Situ Hybridization

Sections of paraffin-embedded specimens were deparaffinized in xylene, rehydrated with ethanol, and subjected to proteinase K digestion (10 μg/ml, 5 min) and 0.2% glycine treatment. Samples were refixed in 4% paraformaldehyde and treated with acetylation solution, rinsing with PBS between treatments. Slides were prehybridized at 53°C for 1 h in hybridization solution (50% formamide, 5× SSC, 0.5 mg/ml yeast tRNA, heparin). Double-DIG LNA-modified DNA probe complementary to mature miR-222 or scramble control (Exiqon) at 40 nM in hybridization solution was incubated overnight at 50°C and washed in SSC at increasing stringency (5 to 0.2× SCC) at 50°C, then with PBST at room temperature. Slides were incubated for 1 h with blocking solution (TBST, 1% BSA, 0.1% FBS) and then 1 h with 1:2,000 dilution of anti-digoxigenin antibody (Roche). After washing with TBST, slides were incubated with AP solution for 15 min and then BM purple-substrate until color development was evident (24 to 72 h). The reaction was stopped when positive controls developed purple color while negative controls remained colorless.

## Transfections

Transfections of 50 nM miR-221, miR-222, miR-29a, miR-200c, and miR-7 mimics (Ambion) were performed as described previously [14]. Protein and RNA were harvested 72 h post-transfection.

## Luciferase Assays

Fragments of the ESR1 3′ UTR containing the putative binding sites for miR-203, miR-221, and miR-22 (nucleotides 2126–2472, ESR1 A) and a region that does not contain any miR-221 or miR-22 binding sites (nucleotides 3585–4249, ESR1 B) were amplified by PCR from HeLa genomic DNA (New England Biolabs). Fragments of the *Dicer1* 3′ UTR containing putative binding sites for miR-29a (nucleotides 1096–1752, Dicer 3′ UTR A) or miR-221/222 (nucleotides 2636–3028, Dicer 3′ UTR B) were also amplified. These fragments were cloned into pMIR-REPORT (Ambion). Site-directed mutagenesis was used to introduce a three-nucleotide mutation into the location where the miRNA see sequence binds (Dicer A mut and Dicer B mut). For ESR1, MCF7s were used, and for Dicer, Hec50 (an endometrial cell line) was used. Cells (20,000) per well were plated into a 96 well plate. The cells were mock transfected, transfected with 50 nM negative control mimic, with mimics for miR-221 or miR-222 (for the ESR1 fragments), miR-29a, miR-222, or antagonists for miR-29a or miR-222 (for the Dicer fragments) (Dharmacon). After 24 h, firefly reporter plasmid (0.196 μg) and a *Renilla* luciferase normalization plasmid pRL-SV40 (0.004 μg) were introduced using Lipofectamine 2000. Cells were harvested 48 h later for analysis using the Dual Luciferase Reporter assay system (Promega).

## Generation of Stable Cell Lines

Cell lines stably expressing shRNAs targeting ZEB1 or luciferase were generated using SMARTvectorTM shRNA Lentiviral Particles (Thermo Scientific Dharmacon) as described previously [15]. For stable expression of the miR-222

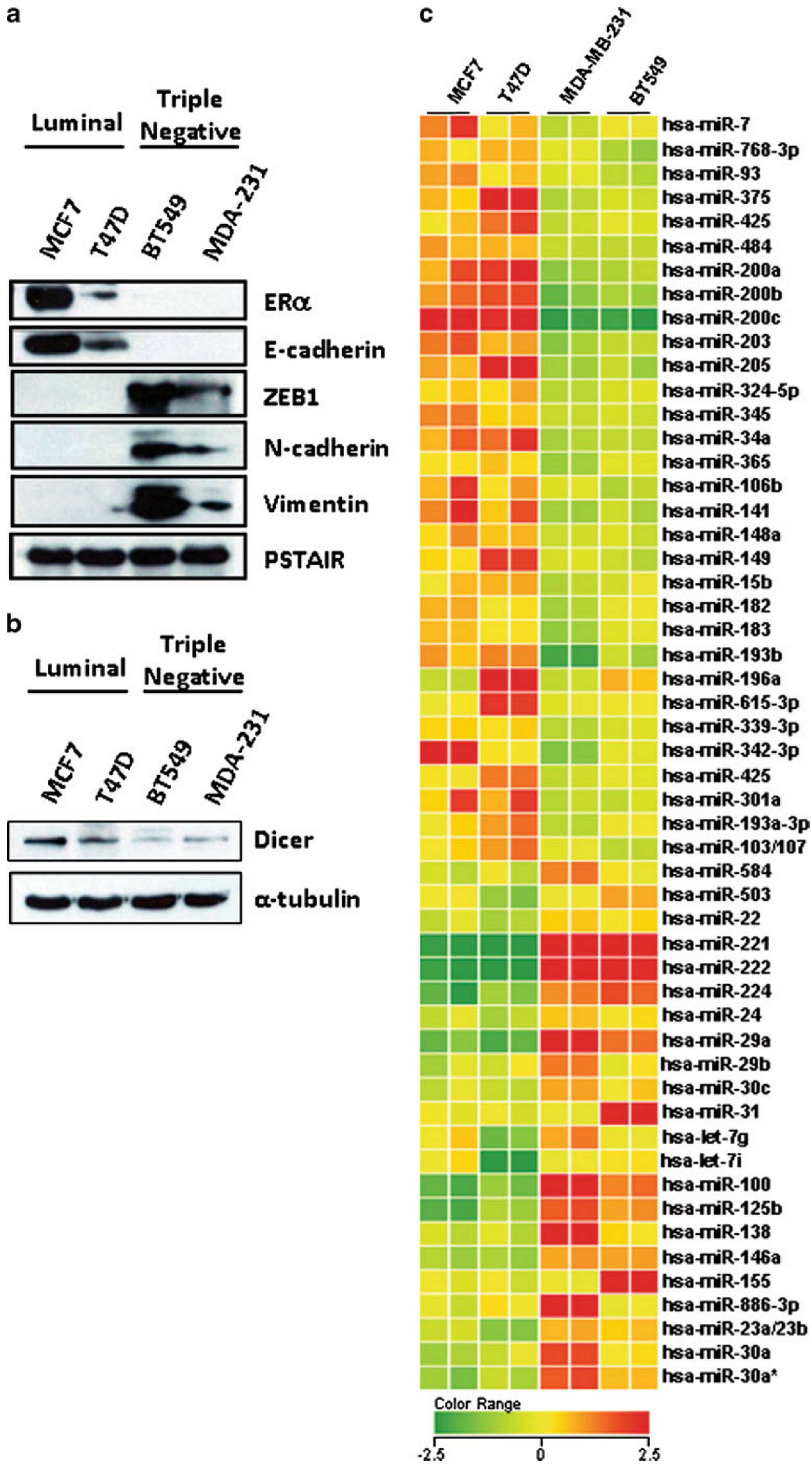



**Fig. 1** MiRNAs differentially expressed in luminal versus triple negative breast cancer cell lines. **a** Protein expression of epithelial markers (ERα, E-cadherin) and mesenchymal markers (ZEB1, vimentin, N-cadherin) in luminal A (MCF7 and T47D) and triple negative (MDA-MB-231 and BT549) cell lines. PSTAIR is shown as a loading control. **b** Immunoblot of Dicer in luminal A (MCF7 and T47D) and triple negative (MDA-MB-231 and BT549) cells, with α-tubulin as a loading control. **c** MiRNA microarray analysis performed in luminal A versus triple negative cell lines. Biological duplicate samples for each cell line were hybridized to Agilent miRNA microarrays. Heatmap of miRNAs that exhibit a 1.5-fold differential expression between luminal and triple negative cell lines

antagonist, pmiR-222-Zip, or pGreenPuro Scramble Control (System Biosciences Inc.), lentiviral vectors were packaged in 293FT cells and virus was harvested after 48 h. Virus was added to MDA-MB-231 or BT549 cells at 1:10 or 1:1 virus: media and selection was performed using puromycin.

## Results

### MiRNAs are Differentially Expressed in ERα+ and ERα− Breast Cancer Cell Lines, the Majority Being More Abundant in ERα+ Cells

We performed miRNA microarray profiling of two breast cancer cell lines representing the luminal A subtype (MCF7 and T47D) and two representing the TN subtype (MDA-MB-231 and BT549). Luminal A cells are relatively well differentiated and retain expression of ERα and E-cadherin, while the TN cells, in particular the basal-like or claudin low subset (which the MDA-MB-231 and BT549 cells represent) [24], have lost expression of these luminal markers and express mesenchymal markers such as ZEB1, N-cadherin, and vimentin (Fig. 1a) and have thus undergone EMT. We also observe that Dicer levels are higher in luminal A cell lines (MCF7 and T47D) compared to TN cell lines (MDA-231 and BT549) (Fig. 1b). Previous reports have indicated that *Dicer1* mRNA expression is lower in carcinoma cells with a mesenchymal phenotype [11, 23]. To determine if this is true in large scale datasets, we mined four breast cancer microarray datasets for *Dicer1* expression separating the data into ESR1+ and ESR1− cohorts. *Dicer1* mRNA levels are significantly lower in the ESR1− breast cancers in all four studies (Supplemental Fig. 1). We find that 53 miRNAs are differentially expressed in luminal A versus TN cell lines (Fig. 1c). Consistent with previous reports that the majority of miRNAs are downregulated in aggressive breast cancers [5, 26, 45], two thirds (31) of the 53 differentially expressed

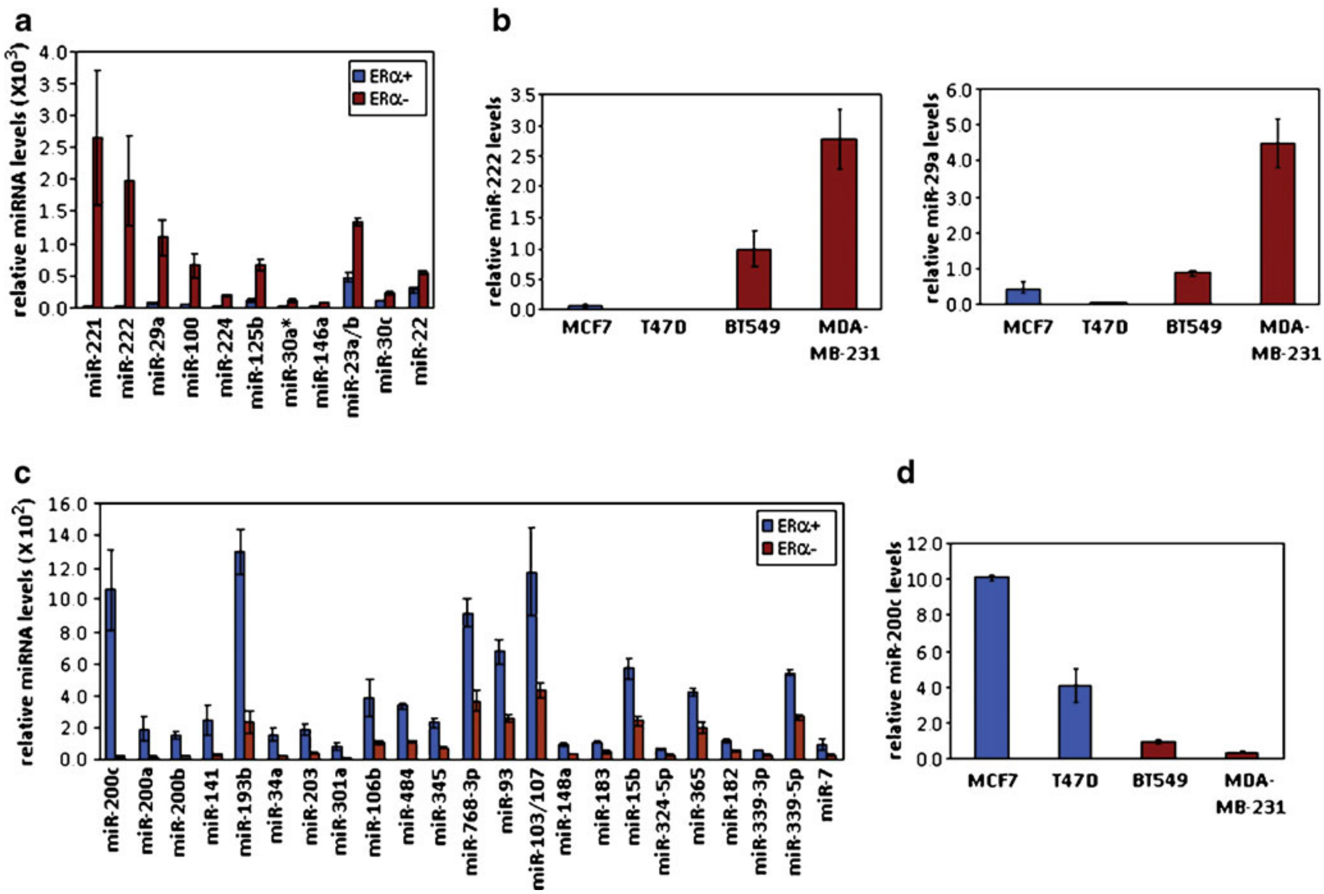



**Fig. 2** MiR-222 and miR-29a are higher in ERα- cell lines compared to ERα+. **a** Graphical representation of miRNAs more highly expressed in ERα+ breast cancer cell lines that are 1.5 different with $P<0.05$. The *blue bars* are the average values for the ERα+ cells (T47D and MCF7), while the *red bars* are the average values for the ERα− cells (MDA-MB-231 and BT549). **b** Real time PCR validation of miR-222 (*left*) and miR-29a (*right*) expression levels, relative to the BT549 values. *Error bars* represent standard error of the mean. **c** Graphical representation of miRNAs more highly expressed in ERα+ cell lines and (**d**) real time PCR validation of miR-200c expression levels, relative to BT549 values. *Error bars* represent standard error of the mean

miRNAs that we identify are higher in ERα+ cells compared to ERα− (Fig. 1c).

### MiR-221/222 and miR-29a are the Most Differentially Expressed miRNAs More Abundant in ERα− Cells

Of the miRNAs higher in ERα− cells, the most differentially expressed and most abundant were the highly homologous miR-221 and miR-222, as well as miR-29a (Fig. 2a). Real time PCR on independent samples confirmed that these miRNAs are more abundant in ERα− (Fig. 2b). The most differentially expressed miRNA that is higher in ERα+ cells is miR-200c, which has been previously demonstrated to be lost in high grade cancers [6, 14, 58], followed by the other miR-200 family members (Fig. 2c). The differential expression of miR-200c in ERα+ and ERα− was also confirmed by RT-PCR (Fig. 2d).

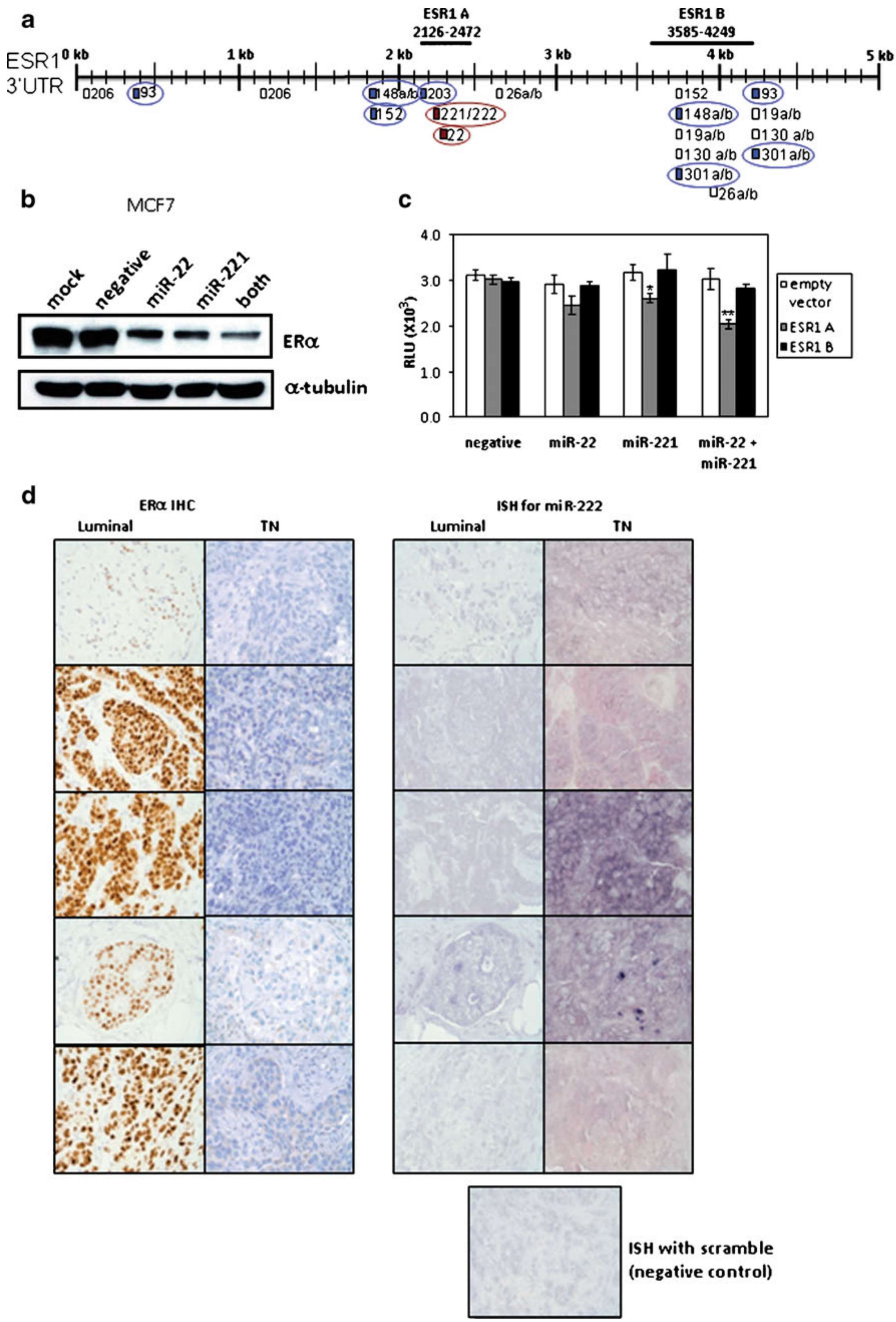
### MiR-222 and miR-22 Act Additively to Decrease ESR1

Study of the miRNAs predicted to target the *ESR1* 3′ UTR using Miranda, PicTar, and Targetscan target prediction programs indicates that the miR-221/222 and miR-22 target sites are in close proximity (Fig. 3a). These miRNAs have been previously reported to target *ESR1* [48, 52, 65], and we find that they are both higher in ESR1− cell lines (indicated in red). In contrast, we find that many of the other miRNAs predicted to bind the ESR1 3′ UTR are paradoxically more abundant in ESR1+ (indicated in blue) (Fig. 3a). MiRNAs can cooperate to downregulate a target when their binding sites are closely located as the miR-221/222 and miR-22 sites are [10, 57]. While the addition of each miRNA alone to ESR1+ MCF7 cells causes a marked decrease in ERα protein, an additive effect was observed when both miRNAs are combined (Fig. 3b). To demonstrate direct targeting of the miRNAs to the 3′ UTR of the *ESR1* transcript, we utilized luciferase reporter assays in which two regions of the *ESR1* 3′ UTR (termed ESR1 A and B) were cloned into the region 3′ of the luciferase gene on a reporter vector. The region denoted ESR1 A contains the miR-221/222 and miR-22 target sites, while ESR1 B is predicted not to be targeted by miR-221/222, miR-22, or any of the miRNA that are higher in ERα− cells and serves as a negative control. Empty luciferase reporter vector containing no target sequences downstream of luciferase also serves as a negative control. These vectors were transfected into MCF7 cells (which lack miR-221/222) in combination with either a scrambled negative control, the miR-22 or miR-221 mimics alone or both miR-22 and miR-221 in combination (Fig. 3c). We observe an 18.8% decrease in luciferase activity in the cells transfected with ESR1 A and the miR-22 mimic compared to the scrambled negative control. With the miR-221 mimic, there is a 13.7% decrease in luciferase activity versus the negative control. When both mimics are combined, we observe a 32.7% decrease in luciferase activity, demonstrating an additive effect when the two miRNAs are combined. We performed in situ hybridization for miR-222 (the homolog of miR-221) on luminal A versus TN (confirmed ERα, progesterone receptor and Her2/neu negative) breast cancer clinical samples obtained from the University of Colorado Breast Cancer Tissue Bank (protocol 04-0066). Figure 3c shows in situ hybridization for miR-222 and IHC for ERα on five representative TN and five luminal A breast cancers. We find miR-222 expression only in TN tumors, whereas in luminal A tumors, miR-222 staining is absent (Fig. 3c). Examples of the levels of staining in cells positive (MDA-MB-231) and negative (MCF7) for miR-222 are shown in Supplemental Fig. 2.

### MiR-221, -222, and miR-29a Target *Dicer1*

In order to test our hypothesis that a direct link exists between miRNAs overexpressed in ESR1− cells and low Dicer levels, we transfected mimics for miR-221/222 and miR-29a into ESR1+ T47D cells and found that they each decrease Dicer protein to almost undetectable levels (Fig. 4a). Real time PCR for each of these miRNAs in the transfected cells is shown in Supplemental Fig. 3. Furthermore, inhibition of miR-222 by stable expression of the antagonist miR-222-ZIP results in increased Dicer protein in both MDA-MB-231 and BT549 cells (Fig. 4b). The *Dicer1* 3′ UTR contains well-conserved predicted target sites for miR-221/222 and miR-29a in close proximity to

**Fig. 3** MiR-22 and miR-221 act additively to decrease ERα levels. **a** Map of 3′ UTR of *ESR1* showing putative miRNA binding sites. Target sites for miRNAs that have higher expression in ESR1− cells are circled in *red*, while target sites for miRNAs more highly expressed in ESR1+ cells are in *blue*. **b** Western blot of MCF7 cells treated with a mock transfection, a scrambled negative control, miR-22 mimic, a miR-221 mimic or a combination of miR-221 and miR-22 mimics. Protein was harvested 72 h after transfection, transferred, and probed for ERα and α-tubulin as a loading control. The experiment was repeated three times; shown is a representative blot. **c** The region of the *ESR1* 3′ UTR containing the miR-22 and 221 binding sites (ESR1 A) and a separate region of the *ESR1* 3′ UTR not containing miR-22 or miR-221 binding sites (ESR1 B) were each cloned downstream of luciferase in a reporter vector. These constructs or the empty reporter vector were transfected into cells treated with a scrambled negative control, miR-22 mimic, miR-221 mimic or both, and a luciferase assay performed. *Error bars* represent standard error of the mean for five replicates. *Single asterisk* indicates a statistically significant difference, $P<0.05$, compared to ESR1 A and *two asterisks* indicate a statistically significant difference, $P<0.01$, compared to EV and ESR1 B (two-way ANOVA, Bonferroni post test). **d** In situ hybridization for miR-222 and immunohistochemistry for ESR1 in luminal and triple negative clinical samples (ESR1 staining is *brown* and miR-222 staining is *purple*). MiR-222 in situ staining with a scrambled negative control is shown at the *bottom* (×400 magnification)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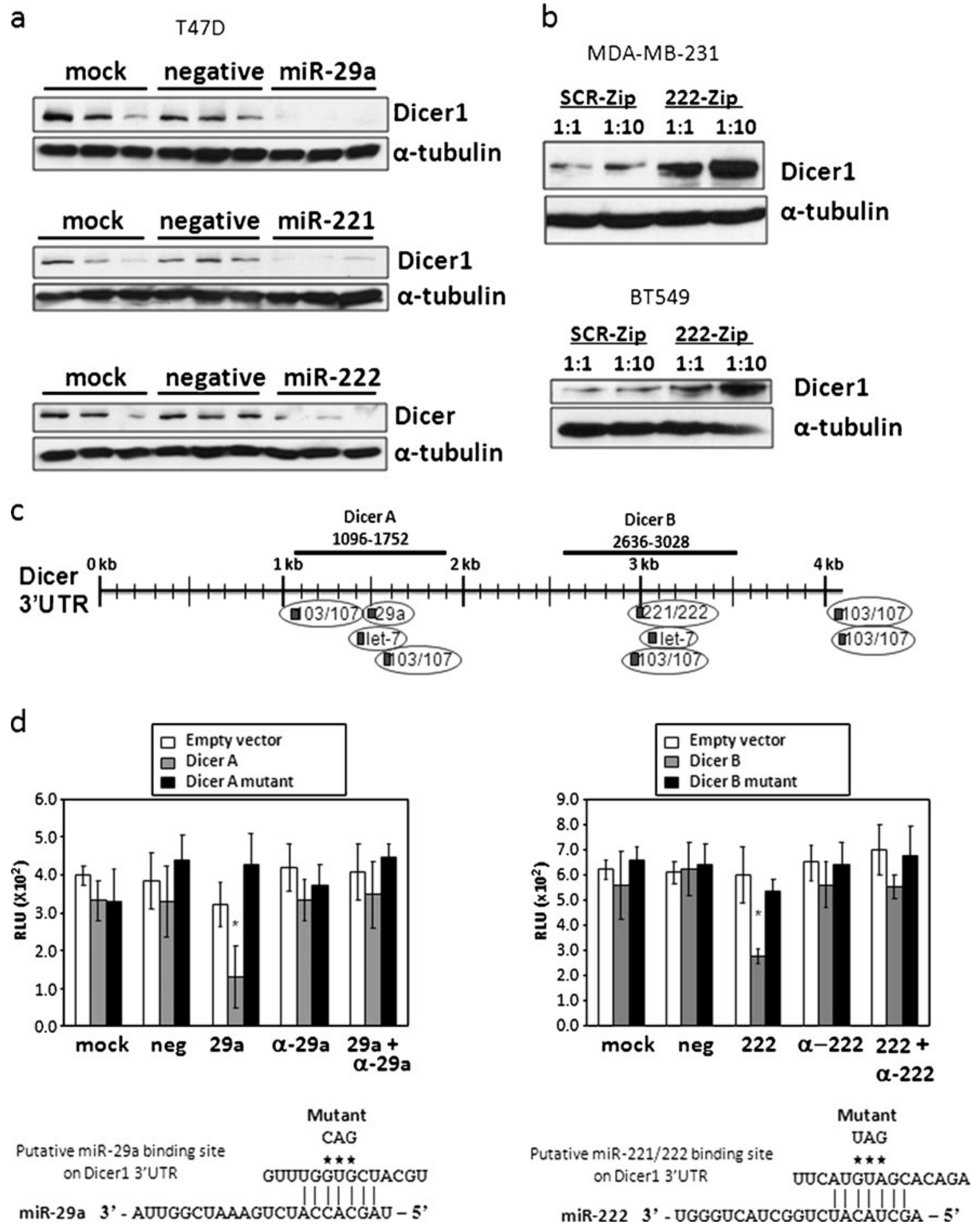


**Fig. 4** MiR-29a and miR-221 or 222 reduce Dicer protein expression by directly targeting Dicer. **a** Immunoblot for Dicer in T47D cells mock transfected, transfected with a scrambled negative control, miR-29a, miR-221, or miR-222 mimic. **b** Immunoblot for Dicer in MDA-MB-231 and BT529 cells stably expressing miR-222 antagonist (222-Zip) or scrambled negative control (SCR-Zip). **c** Map of 3′ UTR of *Dicer1* showing putative miRNA binding sites. Target sites for miRNAs that have higher expression in triple negative cells are in *red*, target sites for miRNAs more highly expressed in luminal cells are in *blue*. **d** Luciferase assay on fragments of the *Dicer1* 3′ UTR containing the miR-29a binding site (*left*) or the miR-221/222 binding site (*right*), fragments containing mutated binding sites or an empty vector. Hec50 cells were mock transfected, transfected with a scrambled negative control, miR-29a or miR-222 mimics, antagonists of miR-29a or miR-222, or a combination of both. The mutations introduced into the putative miRNA binding sites are pictured *below* the graphs. *Asterisk* indicates $P<0.05$, Student's *t* test

previously characterized let-7 sites (Fig. 4c). We cloned two regions of the *Dicer1* 3′ UTR containing the putative miR-29a or miR-221/222 binding sites (Dicer A and B) as well as those same fragments containing mutated miRNA target sites (Dicer A and B mut) downstream of luciferase (Fig. 4c). There is a decrease in luciferase activity only in the cells treated with miR-29a or miR-222 with the appropriate Dicer construct (Fig. 4d). This effect is abrogated when the target site is mutated, showing that the binding site is functional. Furthermore, antagonists of miR-29a and miR-222 are able to prevent binding, showing that the effect is specific to these miRNAs.

## Dicer is Positively Regulated by miR-200c

We previously observed that miR-200c increases *Dicer1* message [14]. We also observed that due to reciprocal repression between miR-200c and ZEB1 [6], reducing ZEB1 expression with shRNA causes an increase in endogenous miR-200c [15]. We find that increasing endogenous miR-200c in MDA-MB-231 cells by using shZEB (which we have shown previously to relieve repression of endogenous miR-200c [15] (Supplemental Fig. 4) or adding exogenous miR-200c mimic increases Dicer protein in MDA-MB-231 and BT549 cells (Fig. 5a, b). Since miRNAs usually function in a repressive manner, the mechanism by which miR-200c increases Dicer protein is likely through an indirect mechanism. We hypothesized that since many mature miRNAs are low in ERα− cells (perhaps due to inefficient maturation as a result of low Dicer), increasing Dicer might increase levels of mature miRNAs typically low in the TN cells. To test this hypothesis we measured levels of the mature forms of miRNAs originally observed to be low in TN cells in cells transfected with miR-200c mimic (in which endogenous Dicer levels had increased). We find that in MDA-MB-231 cells, miR-193b, miR-34a, and miR-148a are increased with miR-200c mimic compared to the negative control (Fig. 5a). Several other miRNAs (miR-15b, miR-103, miR-301a, and miR-106b), which we also find to be more abundant in ERα+ cells, also demonstrated increased levels in the miR-200c treated cells (data not shown). In BT549 cells, we observe an increase in miR-34a, miR-148a, and miR-301a when transfected with the miR-200c mimic (Fig. 5b). However, addition of miR-200c does not repress miR-221/222 levels (data not shown).

## MiR-7 is an Estrogen-Regulated miRNA that Targets Growth Factor Receptors Overexpressed in TN Breast Cancers

To identify miRNAs not only associated with ERα positivity, but actually regulated by estradiol-bound ERα, we performed miRNA microarray profiling of MCF7 cells treated for 24 h with 10 nM estradiol or ethanol vehicle control (Fig. 6a). At 24 h, the expression of six miRNAs significantly decreased while eight significantly increased with estrogen treatment. MiR-7 and miR-324-5p are both

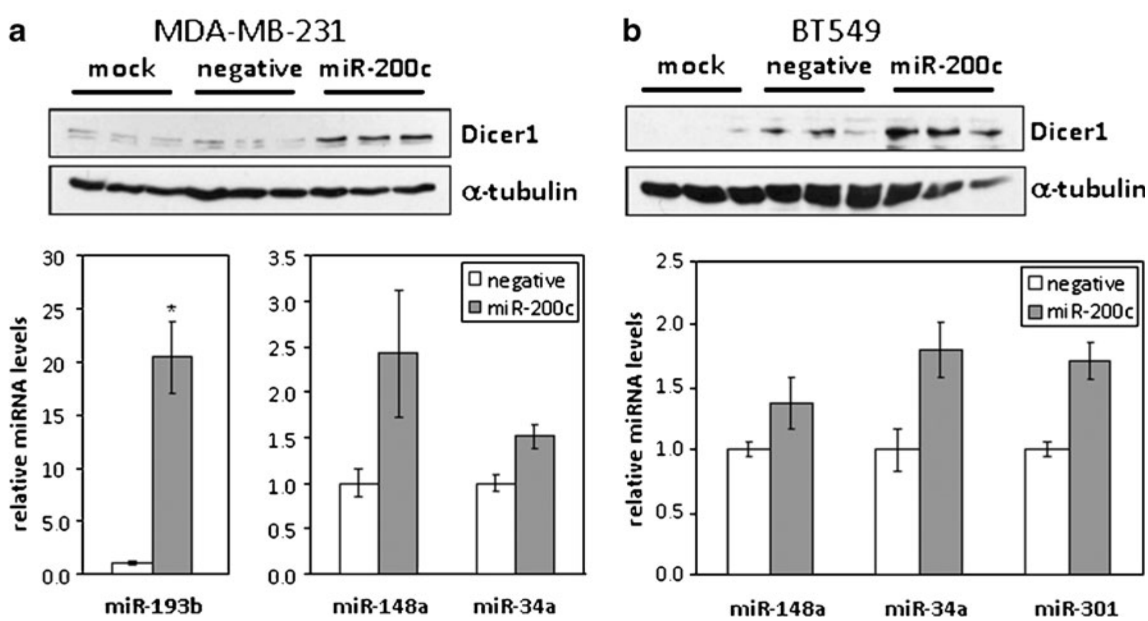


**Fig. 5** Restoration of miR-200c to ESR1− breast cancer cells increases Dicer protein. Immunoblot for Dicer in MDA-MB-231 (**a**) and BT549 (**b**) cells mock transfected, transfected with a scrambled negative control or a miR-200c mimic for 72 h. Bottom, real time PCR for miR-193b, miR-34a, and miR-148a in MDA-MB-231 cells and mR-34a, miR-148a, and miR-301a in BT549 cells transfected with a scrambled negative control or a miR-200c mimic. An *asterisk* indicates $P<0.05$, Student's *t* test

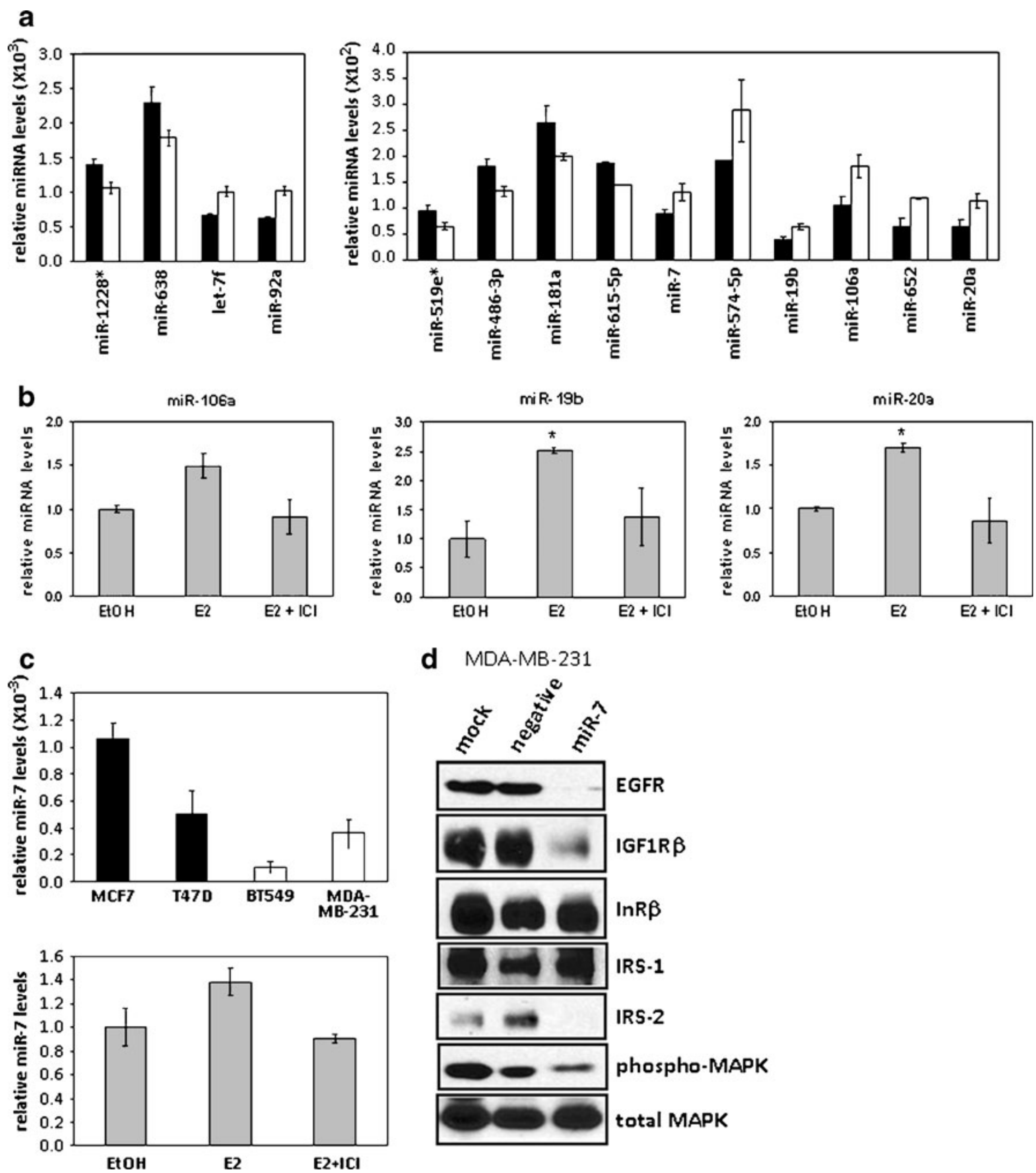


**Fig. 6** MiR-7, which is associated with ERα positivity and is upregulated by estrogen, targets growth factor receptors and downstream signaling molecules. **a** MiRNA microarray analysis of miRNAs differentially regulated by 10 nM estradiol (*white bars*) at 24 h versus the ethanol vehicle controls (*black bars*) in MCF7 cells. Shown are the miRNAs that have a 1.5-fold difference and $P<0.05$. *Error bars* represent the range of biological duplicates. **b** Real time PCR for miR-106a, miR-19b, and miR-20a in MCF7 cells treated with the ethanol vehicle control, 10 nM estradiol (E2) or estradiol, and 1 μM ICI (E2+ICI) for 24 h. Shown are the averages of three replicate samples, and *error bars* represent standard error of the mean. *Asterisk* indicates a statistically significant difference between E2 treated and the vehicle control, with $P<0.05$, Student's *t* test. **c** Real time PCR for miR-7 was performed in two ESR1+ cell lines (MCF7 and T47D) and two ESR1− cell lines (BT549 and MDA-MB-231), *top*, and in cells treated with the ethanol vehicle control, 10 nM estradiol (E2) or estradiol, and 1 μM ICI (E2+ICI) for 24 h, *bottom*. Shown are the averages of three replicate samples, and *error bars* represent standard error of the mean. **d** MDA-MB-231 cells were mock transfected, transfected with a scrambled negative control, or a miR-7 mimic for 72 h. Protein was harvested and blots probed for EGFR, IGF1Rβ, InRβ, IRS-1, IRS-2, phospho-MAPK ,and total MAPK (also used as a loading control)

higher in ESR1+ cells and positively regulated by estrogen. We confirmed in independent samples by real time RT-PCR that several members of the miR-17-92 cluster or the paralog miR-106a-363 cluster are estrogen regulated (Fig. 6b). MiR-20a and one of the copies of miR-19b appear in the miR-17-92 cluster, while miR-106a and the other copy of miR-19b are in the miR-106a-363 cluster. We confirm that miR-7 is expressed more highly in ESR1+ cell lines and estrogen increases miR-7 levels in an ESR1-dependent manner (Fig. 6c). Bioinformatic analysis predicts epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), and insulin receptor substrates 1 and 2 (IRS-1, IRS-2) as putative miR-7 targets. IGF1R contains three predicted miR-7 binding sites and IRS-2 contains two putative binding sites. Addition of a miR-7 mimic to ESR1− cells dramatically decreased EGFR and IGF1Rβ at the protein level with no effect on the insulin receptor (Fig. 6d). There is also a profound decrease in IRS-2 protein following the addition of the miR-7 mimic, but no effect on IRS-1. Finally, we observe a decrease in the amount of phosphorylated ERK1/2 (MAPK) with no effect on total MAPK.

A schematic of the regulation of key distinguishing features of TN versus luminal cancers by miRNAs is shown in Fig. 7. Both Dicer and ERα are expressed at high levels in luminal breast cancers and are markers of a differentiated epithelial phenotype. MiR-221 and miR-222 are high in TN breast cancers and target both Dicer and ERα. MiR-29a is also high in TN breast cancers and targets Dicer. MiR-200c is high in luminal breast cancers and increases Dicer expression. MiR-7 is expressed at high levels in luminal A cells and limits the expression of growth factors receptors such as EGFR and IGF1R that are often overexpressed in TN cancers, and the signaling intermediate IRS-2.

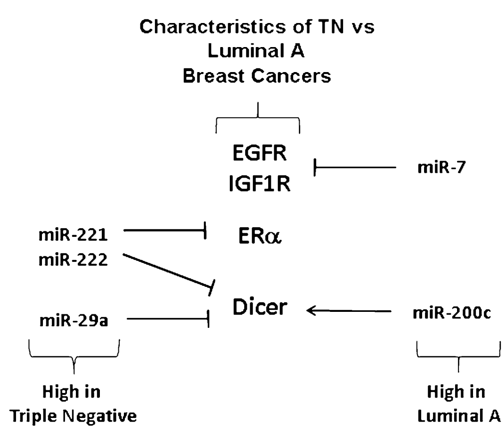


**Fig. 7** MiRNA regulation of key proteins in luminal and triple negative breast cancers. Dicer and ERα are expressed at high levels in luminal breast cancers. MiR-221 and miR-222 are high in TN breast cancers and target both Dicer and ERα. MiR-29a is also high in TN breast cancers and targets Dicer. MiR-200c is high in luminal breast cancers and increases Dicer expression. EGFR, IGF1R, and IRS-2 are often activated or overexpressed in TN cancers and are all targeted by miR-7

## Discussion

MiRNA profiling of ERα+ versus ERα− breast cancer cell lines reveals that the majority of miRNAs are lower in the ERα− cells. This is consistent with previous reports of a global decrease in miRNA expression in cancer [5, 26, 45]. While many miRNAs are located in fragile sites that are often lost in cancer [7], it is also possible that decreased expression of component(s) of the miRNA processing machinery prevent efficient miRNA processing. Indeed, cancer cells can have decreased expression of mature miRNAs, while maintaining expression of precursors [39, 47, 59] and impairment of miRNA processing results in transformation and increased tumorigenesis [36, 37]. MiRNAs are essential for differentiation and maintenance of a differentiated state. Dicer-deficient stem cells are unable to properly differentiate [27, 28], and loss of Dicer causes apoptosis in differentiated neural crest cells [63] and prostate epithelial cells [64]. The loss of differentiation and increased aggressive behavior that accompanies EMT may be in part due to decreased Dicer expression and a resultant decrease in mature miRNA expression.

The data presented herein as well as that of others [11, 23] suggests that high Dicer levels in breast cancer are associated with a well-differentiated epithelial, ERα+ phenotype, while lower Dicer levels are found in the less differentiated ERα- cells. Furthermore, Dicer has been shown to be positively regulated by estradiol [1]. Our findings demonstrate that while the majority of miRNAs are more abundant in ERα+ cells, miR-221/222 and miR-29a are striking exceptions. ESR1 is directly targeted by miR-221/222 and miR-22 [52, 65], and we demonstrate that these miRNAs cooperate to decrease ERα. In clinical samples, we find that expression of miR-222 and ERα is mutually exclusive, consistent with previous reports of miR-222 repressing ESR1 [48, 65] and the reciprocal negative regulatory loop whereby ERα represses miR-222 [16].

We show that miR-221/222 and miR-29a directly target *Dicer*, and these miRNAs are likely responsible for repressing Dicer expression and function in ERα breast cancer. We find that let-7 is higher in ERα breast cancer cells, and it also directly targets and represses *Dicer1* [19, 60]. Since there are let-7 sites in close proximity to both the miR-221/222 and miR-29a binding sites, it is possible that these miRNAs work cooperatively.

MiR-200c represses a program of mesenchymal genes to maintain an epithelial state [6, 14, 15, 22, 25, 34], and here, we show that it also positively regulates Dicer, likely through an indirect, yet to be identified mechanism. Importantly, this may represent an additional means by which miR-200c promotes a well-differentiated epithelial phenotype. Our studies indicate that a subset of miRNAs may be low due to insufficient Dicer. While lower levels of mature miRNAs in ERα− cells can be explained by decreased Dicer levels, this begs the question as to how some miRNAs (such as miR-221/222 and miR-29a) are abundant in ERα− breast cancers in the face of low Dicer expression. In lower organisms, such as *Drosophila*, there are two Dicer proteins; however, in humans, only one Dicer gene exists (*Dicer1*). Not all miRNAs are equally affected by Dicer depletion [21, 35], suggesting either that miRNA stability is a factor, or perhaps another enzyme exists that can process certain miRNA precursors when Dicer levels are low. For example, miR-451 can be fully processed by Ago2 [9, 13], which is higher in ERα− breast cancers [11] and personal communication (Dorraya El-Ashry and Phillip Miller).

Since both *Dicer* and *ESR1* and their protein products are low or absent in TN breast cancer cells, it makes sense that both are targeted by miRNAs abundant in TN cells. However, both the *ESR1* and *Dicer* 3′ UTRs also have putative target sites for miRNAs that are highly expressed in ERα+ cells. It is likely that other factors are interfering with the miRNA–mRNA interaction. For instance, RNA-binding proteins can bind 3′ UTRs and prevent or recruit miRNA binding [3, 4, 29, 30] or target sites can be mutated or absent due to shortening of the 3′ UTR [20, 46, 50, 55]. Non-coding RNAs or pseudogenes can act as decoys to soak up miRNAs and prevent them from interacting with a target [54, 61]. For instance miR-193b (5.5-fold higher in ESR1+ cells in our study) directly targets *ESR1* when it is transfected into MCF7 cells [40]. Perhaps overexpression of this miRNA can overcome whatever is preventing the already abundant endogenous miR-193b from targeting *ESR1*. Similarly, miR-103/107 was recently reported to directly target *Dicer1* [44]. However, in our study and others [42, 45], miR103/107 is higher in ERα+ cells (which have high Dicer) as compared to ERα- cells. While miR-193b and miR-103/107 can target ESR1 and Dicer if overexpressed, these miRNA are already expressed at higher levels in ERα+ cells that express substantial Dicer. Nevertheless, it is possible that these miRNAs naturally fluctuate under certain conditions in order to fine tune or limit ERα or Dicer protein levels.

We sought to determine if any of the miRNAs differentially expressed in ERα+ versus negative breast cancer cells are differentially expressed because they are regulated by estradiol-bound ERα [2, 8, 32]. Several miRNAs located in the miR-17-92 cluster or its paralog clusters are upregulated by estrogen. The miR-17-92 cluster (also known as *oncomir-1*) has been implicated in several types of cancers [12, 17, 51]. MiR-7 was also both estrogen regulated and more abundant in ERα+ cells. MiR-7 targets EGFR and decreases proliferation [41, 62]. We further demonstrate that miR-7 can also reduce IGF1R and IRS-2 protein expression. EGFR and IGF1R are often overexpressed and constitutively active in TN breast cancers and contribute to an aggressive phenotype [33, 38]. Similarly, IGF1R is often activated in aggressive cancers with poor prognosis, and overexpression of IGF1R in a mouse model results in mammary gland tumors with a basal-like phenotype [31]. Since IRS-2 is a signaling intermediate in the IGF1R pathway [49, 53], miR-7 could be a very effective means by which to abrogate this pathway. Our data suggest that effective re-introduction of miR-7 into TN breast cancer could offer an advantage over inhibitors targeting either EGFR or IGF1R since it would target both pathways simultaneously.

In summary, we demonstrate that the most highly differentially expressed miRNAs more abundant in ERα− breast cancers, namely miR-221/222 and miR-29a, directly repress *Dicer1*. In contrast, miR-200c, which is more abundant in ERα+ breast cancer cells, increases Dicer protein levels. We conclude that miRNAs differentially expressed in ERα+ versus negative breast cancer cells function to control some of the most distinguishing characteristics of the luminal A as compared to TN breast cancer subtypes such as ERα status, Dicer protein levels, and EGFR and IGF1R growth factor receptor expression.

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**Conflict of Interest** The authors declare that they have no conflict of interest.

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# microRNAs and EMT in Mammary Cells and Breast Cancer

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**Abstract** MicroRNAs are master regulators of gene expression in many biological and pathological processes, including mammary gland development and breast cancer. The differentiation program termed the epithelial to mesenchymal transition (EMT) involves changes in a number of microRNAs. Some of these microRNAs have been shown to control cellular plasticity through the suppression of EMT-inducers or to influence cellular phenotype through the suppression of genes involved in defining the epithelial and mesenchymal cell states. This has led to the suggestion that microRNAs maybe a novel therapeutic target for the treatment of breast cancer. In this review, we will discuss microRNAs that are involved in EMT in mammary cells and breast cancer.

**Keywords** microRNA · Epithelial to mesenchymal transition · Mammary cells · Breast cancer

**Abbreviations**

| | |
|---|---|
| miRNA | microRNA |
| EMT | epithelial to mesenchymal transition |
| MET | mesenchymal to epithelial transition |
| TGF-β | Transforming Growth Factor β |
| HMEC | human mammary epithelial cell |
| bCSC | breast cancer stem cell |

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## microRNAs

MicroRNAs (miRNAs) are non-coding RNAs that regulate gene expression at the post-transcriptional level by inhibiting translation or initiating mRNA destruction. There is increasing evidence that miRNAs maybe master regulators of many fundamental biological processes, such as embryogenesis [1] and organ development [2]. Individual miRNAs can enforce a developmental switch or tissue-specific gene expression through regulation of a key mRNA target [3, 4] or through many mRNA targets [5]. Similar to other forms of gene regulation, miRNAs have been shown to be involved in human cancers, acting as oncogenes [6, 7] or tumor suppressors [8, 9]. Therefore, understanding this form of gene regulation maybe a key to regulating fundamental biological processes and controlling disease progression.

Some miRNAs are expressed in a cell-specific, tissue-specific and/or developmental stage-specific manner, while others are expressed relatively ubiquitously. As with other genes, this is dependent upon the transcription factors that regulate their expression. MiRNA genes are transcribed by RNA polymerase II as their own dedicated transcript [10] or can be cleaved from introns of protein-coding genes. Cleavage of the primary transcript by the DROSHA/DGCR8 complex [11] generates a 60–70 nucleotide (nt) stem-loop pre-miRNA, which is exported from the nucleus by the exportin 5-RanGTP system [12]. Within the cytoplasm, the RNAse III enzyme Dicer processes the pre-miRNA to yield the 18–24 nt mature miRNA [13, 14]. Through imperfect base pairing, miRNAs bind to target mRNAs in the context of the RNA-induced silencing complex (RISC) [15]. Complementarity between nucleotides 2–8 at the 5′ end of the miRNA (termed the "seed sequence") is important for efficient targeting. While a single miRNA can be encoded in one primary transcript, often a cluster of multiple miRNAs are encoded in a polycistronic transcript. Differences in the seed sequences of miRNAs within a cluster means that a single cluster of miRNAs has the potential to regulate an enormous range of targets.

## miRNA Regulation of EMT

Epithelial to mesenchymal transition (EMT) is involved in embryonic development, wound healing and cancer progression (reviewed in [16]). During an EMT, epithelial cells lose cell-cell contacts and undergo cytoskeletal remodelling and polarity changes, resulting in acquisition of mesenchymal morphology and enhanced migratory ability. Importantly, the process of EMT is reversible (termed a mesenchymal to epithelial transition or MET), so that polarised epithelium can be generated in a new site. Many signalling pathways induce EMT, including Transforming Growth Factor β (TGF-β), Wnt and Notch, in part through regulation of several transcription factors, including Snail1/2, E47, Klf8, ZEB1/2 and Twist (reviewed in [16]). A key target of these EMT-inducing transcriptional repressors is the epithelial-specific junctional protein, E-cadherin. Loss of epithelial specific proteins, such as E-cadherin, and increased expression of mesenchymal specific proteins, such as vimentin, can be used as markers to show that an epithelial cell has undergone an EMT. Here we highlight miRNAs that are regulated by and control EMT (Fig. 1).

Microarray analysis has been used to identify miRNAs that are involved in EMT, comparing cells before and after induction of EMT. When EMT is induced with different

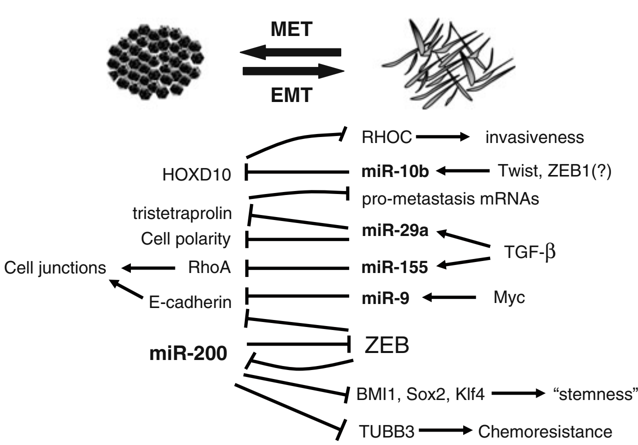


**Figure 1** Simplified overview of microRNAs involved in EMT (refer to text for details and references).

stimuli, the most consistently striking change is the reduction in levels of the miR-200 family and miR-205 [4, 17–20]. The miR-200 family include two clusters of miRNAs — miR-200b~200a~429 and miR-200c~141 [4]. The dramatic decrease in miR-200 family and miR-205 expression with EMT is reflected in lower expression of the miR-200 family and miR-205 in mesenchymal cell lines compared to epithelial lines [4, 20–22]. Significantly, these correlations have led to the discovery that the miR-200 family are key regulators of EMT. Through their suppression of the mesenchymal-specific repressors of E-cadherin transcription, ZEB1 and ZEB2, the miR-200 family increase the levels of E-cadherin and are capable of enforcing the epithelial phenotype in mesenchymal cells [4], including mesenchymal-like, MDA-MB-231 breast cancer cells [17, 20]. Additionally, their expression in epithelial cells can inhibit TGF-β1-induced EMT in canine kidney and murine mammary epithelial cell lines, MDCK and NMuMG cells respectively [4, 22]. Conversely, inhibition of the miR-200 family in epithelial cells induces EMT [4, 17, 20]. In part, the reversibility of EMT is determined by the fact that ZEB1 and ZEB2 suppress miR-200 family expression [23]. This double negative feedback loop enables the miR-200 family and ZEBs to respectively maintain the epithelial and mesenchymal cellular states. Additional EMT-related targets of the miR-200 family have also been reported, including TGF-β2 by miR-141 [17] and Beta-catenin (CTNNB1) by miR-200a [24]. Given that miRNAs are capable of regulating hundreds of miRNA targets and the key role the miR-200 family play in enforcing the epithelial phenotype, further miR-200 family targets that are involved in EMT may still be identified.

The key role that the TGF-β signalling pathway plays in EMT and cancer (reviewed in [25]), has prompted studies focussed upon identifying miRNAs involved in TGF-β-induced EMT. In contrast to the miR-200 family, levels of miR-155 are increased during TGF-β-induced EMT in mammary epithelial cell model systems, via transcriptional activation by SMAD4 [19]. While ectopic expression of miR-155 did not induce an EMT, cell polarity and tight junction formations were disrupted and cells responded more rapidly to TGF-β. Significantly, loss of miR-155 suppresses TGF-β induced EMT in NMuMG cells. A key miR-155 target is RhoA, which plays an important role in formation and stabilisation of cell junctions [19]. In addition to miR-155 regulation of RhoA, TGF-β induces the ubiquitination and degradation of RhoA by Smurf1 E3 ligase, in response to activation by Par6 [26, 27]. Accordingly, miRNAs such as miR-155 provide a further layer of regulation that ensures the suppression of specific proteins to control cellular phenotype.

MiR-29a and miR-21 levels increase upon TGF-β-induced EMT in mammary epithelial model systems [18, 19] and are higher in many mesenchymal cell lines compared to epithelial cell lines [20], although the precise relationship between these miRNAs and EMT has not been extensively explored. Significantly, suppression of tristetraprolin (TTP) by miR-29a induces EMT in oncogenic Ras-expressing murine mammary epithelial cells [18]. However, since the overexpression of miR-29a in non-tumorigenic murine mammary epithelial cells did not induce an EMT, the relationship between miR-29a and EMT is clearly context dependent. In contrast, there are no reports of miR-21 directly regulating EMT. However, there have been multiple reports of pathways regulating miR-21 in response to TGF-β. Pre-miR-21 and mature miR-21 levels are increased by TGF-β in MDA-MB-468 breast carcinoma cells via a SMAD4-independent, post-transcriptional mechanism, involving increased processing of the miR-21 primary transcript by the Drosha complex [28, 29]. Additionally, miR-21 transcription can be activated by the TGF-β-regulated transcription factor, AP-1 [30, 31] or by the EMT-inducer, ZEB1 [32, 33]. The functional significance of increased levels of miR-21 with EMT maybe elucidated from its target mRNAs, which have roles in EMT, cell cycle control and apoptosis (TGFβR2, DAXX, Cdc25A, PDCD4, TPM1, PTEN) [34–39]. Further exploration of the role of these miRNAs in EMT is required.

EMT can also be induced by the transcription factor Twist [40]. Amongst other genes, Twist activates miR-10b transcription through an E-box proximal to the predicted promoter [41]. In contrast to Twist, miR-10b alone cannot induce an EMT. However, miR-10b increases motility and invasiveness of HMECs (human mammary epithelial cells) and breast cancer cells and contributes to the migratory and invasive properties conferred by Twist. It is still unclear whether miR-10b is specifically related to Twist or is involved in EMT induced by other stimuli. There is some evidence that other E-box binding EMT-inducers may have opposing effects on miR-10b. For example, Snail1 reduced miR-10b expression in HMECs [41]. Microarray data suggest that ZEB1 may increase miR-10b levels in colorectal cancer cells, but decrease miR-10b levels in breast cancer cells [17], suggesting that the link between miR-10b and EMT is context dependent.

Loss of E-cadherin can induce EMT in epithelial cells [42]. Accordingly, miRNAs that affect E-cadherin expression are likely to be regulators of EMT. miR-9 has recently been reported to suppress E-cadherin expression and induce EMT in immortalised human mammary epithelial (HMLE) cells [43]. Interestingly, miR-9 cannot induce EMT in an epithelial breast cancer cell line, SUM149, in vitro, however, there is increased expression of the mesenchymal marker, vimentin, at the tumour-stroma interface in vivo compared to control, which suggests that miR-9 may sensitise cells to signals from the tumour microenvironment that induce EMT.

## miRNAs and Mammary Stem Cells

Mammary development and homeostasis is thought to be dependent upon a differentiation hierarchy, with mammary stem cells at the apex (reviewed in [44]). Cell populations enriched in mammary stem cells can be prepared by dissociating mouse mammary tissue into single cell suspensions and fractionating the cells according to cell surface markers, such as CD24$^{med}$/CD49f$^{hi}$ and CD29$^{hi}$/CD24$^{+}$ [45, 46]. Mouse mammary stem cells isolated by these methods are able to reconstitute entire mammary glands in vivo [45, 46]. In an effort to understand molecular pathways regulating stem cell properties, microarray studies were performed to assess differences in mRNAs and miRNAs in mammary stem cells compared to mammary progenitor and mature cells. Higher levels of the mesenchymal marker, vimentin, and lower levels of E-cadherin and the miR-200 family are found in human mammary stem cells (CD49f$^{hi}$EpCAM-) compared to luminal progenitor and mature luminal cells (CD49f$^{-/+}$EpCAM+) [47, 48]. Similarly, enriched populations of mouse mammary stem cells (CD24$^{med}$CD49f$^{hi}$) have lower levels of the miR-200 family compared to more differentiated mammary epithelial progenitor cells (CD24$^{hi}$CD49f$^{lo}$) [48]. According to the expression of these epithelial and mesenchymal markers, differentiation of mammary stem cells may follow a program similar to a MET. Importantly, the low levels of the miR-200 family in mammary stem cells are significant. This is most clearly demonstrated when miR-200c is ectopically expressed in murine mammary cells isolated from FVB/NJ mice. In contrast to controls, when miR-200c expressing cells are injected into the cleared mammary fat pad of FVB/NJ mice, normal mammary outgrowth is suppressed and myoepithelial differentiation is induced [48]. This suggests that the miR-200 family may regulate differentiation of mammary stem cells.

In contrast to the miR-200 family, significantly higher expression of miR-205 is observed in enriched populations of [1] basal and myoepithelial stem-cells (CD24$^{+/lo}$/Sca-1$^{-}$) compared to luminal (CD24$^{+/hi}$/Sca-1$^{-/+}$)cells, [2] self-renewing stem cells (CD29$^{hi}$/CD24$^{+}$) compared to non-stem cells and [3] stem cells (CD24$^{med}$/CD49f$^{hi}$) or myoepithelial cells (CD24$^{lo}$/CD49f$^{lo}$) compared to non-stem cells (CD24$^{+/hi}$/CD49f$^{-}$) [49]. The high levels of miR-205 in mammary stem cells are functionally significant, because when miR-205 was ectopically expressed in a cell line model of mammary gland progenitor cells, there was a significant expansion of the progenitor population [49, 50]. Despite the similar roles of the miR-200 family and miR-

205 in regulating ZEB expression and EMT [4], differences in the response of mammary stem cells to these miRNAs highlights the differences in the range of targets regulated by these miRNAs. Other miR-205 targets have been identified, including PTEN, protein kinase Cε, LRP1 and HER3 [49, 51–53], as well as many other potential targets predicted from a microarray conducted on miR-205-expressing cells [49]. Further experiments are required to fully understand the role of miR-205 in mammary stem cells, development and tissue homeostasis.

## miRNAs, EMT and Mammary Gland Development

While there are reports of miRNA involvement in mammary gland development [54, 55], there is very little discussion of EMT in mammary gland development. However, a process reminiscent of wound healing and EMT occurs between lactation and involution. Mammary involution is initiated at the end of lactation, which involves cessation of milk secretion, massive cell death, collapse of the alveoli, clearance of apoptotic cells and remodelling of the epithelial compartment to restore a simple ductal structure (reviewed by [56, 57]). By day 4 of involution, TGF-β signalling pathways increase [58–60], coinciding with decreased levels of E-cadherin [61, 62]. miRNA expression profiles suggest that miRNAs may play a role in this EMT-like process in late involution, with decreased levels of miR-200a, miR-429 and miR-141 and increased expression of miR-29a, miR-21, and miR-10b [54]. Similar to the changes observed in mammary progenitor cells [49, 50], changes in miR-205 levels are in direct contrast to the miR-200 family. However, the precise roles of EMT and associated miRNAs in mammary gland development have yet to be explored.

## miRNAs, EMT and Breast Cancer Metastasis

Metastasis is the most common cause of death for breast cancer patients. Evidence for the involvement of miRNAs in the metastatic process is rapidly accumulating, presenting an attractive novel therapeutic possibility. Altered expression of the miRNA processing machinery is observed in more invasive, aggressive breast cancers. For example, levels of Dicer are lower in mesenchymal compared to epithelial breast cancer cell lines and also in the more aggressive basal-like, HER2+ and luminal B type tumours compared to luminal A type breast cancer [63–65]. The latter maybe linked to the frequent hemizygous deletion of Dicer associated with breast tumors [66]. Specific miRNAs have also been directly linked to metastasis. For example, miR-31, miR-373, miR-520c, miR-126 and miR-335 potentially regulate metastasis through a range of mRNA targets [67–70].

One theory to explain how metastases arise from a primary breast tumor is that peripheral epithelial cells receive signals from the surrounding stroma to undergo the EMT program, thus enhancing tumor cell motility and invasiveness (reviewed in [16]). This hypothesis is supported by studies showing a loss E-cadherin and higher levels of mesenchymal markers in invasive ductal, basal-like and metaplastic carcinoma of the breast [71–73]. The links between metastasis and EMT are also reflected in the expression of EMT-related miRNAs. MiR-21, miR-9 and miR-155 levels are increased in malignant breast cancer and breast cancer cell lines compared to normal tissues and human mammary epithelial cells [74–76]. Both basal and metaplastic breast cancers have reduced expression of the miR-200 family compared to ductal breast tumors, which correlates with their invasiveness [4, 17]. This contrasts miR-205 levels, which are not significantly different between ductal and metaplastic primary breast carcinomas [4], once again highlighting differences in the mRNA targets of between the miR-200 family and miR-205. However, direct links between these EMT-related miRNAs and metastasis are most clear upon comparison of primary tumour samples to metastases — higher levels of miR-10b, miR-21 and miR-155 and lower levels of the miR-200 family were observed in metastatic samples compared to matched primary tumours [77]. These observations have led to the hypothesis that manipulation of the EMT program through EMT-regulating miRNAs may limit metastasis.

Given the key role that the miR-200 family plays in regulation of the epithelial phenotype and EMT, it has been hypothesized that enforced expression of these miRNAs will limit metastasis [78]. In support of this hypothesis, knockdown of the key miR-200 target ZEB1 in Panc1 cells decreases primary pancreatic tumour size and inhibits local infiltration and metastasis upon intrapancreatic injection into nude mice [79]. A role for miR-200 in suppressing metastasis is supported by work with a lung adenocarcinoma model, where miR-200 family expression in metastasis-prone cells did not affect primary tumor growth rate but inhibited metastasis from tumors formed by subcutaneous injection of the cells into the posterior flank of 129Sv mice [80]. The potential for the miR-200 family to suppress metastasis is also inferred from other identified miR-200 family targets, leptin receptor and cofilin 2, which are known promoters of metastasis [17]. This suggests that the miR-200 family maybe capable of limiting metastasis through suppression of a range of mRNA targets involved in multiple pathways. Numerous studies have found that miR-200 inhibits migration or invasion of cells in vitro. This includes MDCK cells [4], 344SQ lung adenocarcinoma cells [80], NPC nasopharyngeal carcinoma cells [24], SW480 colorectal cancer cells [17], Hec50 endometrial cancer cells [81], ovarian cancer SKOV-3 cells [82], LNCaP prostate cancer cells [83], TGF-β1 treated MCF10A breast cancer cells [84], MDA-MB-231 cells [17, 20, 85] and 4T07 breast cancer cells [22]. This latter result with 4T07 cells is contradicted by a report that ectopic expression of miR-200c~141 in 4TO7 cells increased migration, despite the cells having undergone an MET (according to their epithelial-like morphology, loss of ZEB2 and gain of E-cadherin) [86]. One difference between the two studies is that the latter investigated migration through membranes coated with a mixture of basement membrane components whereas the former study used uncoated membranes, although it is not clear that this accounts for the differences in effect of miR-200 on migration, especially since other reports show miR-200 reducing invasion through matrigel [17, 24, 80, 81, 83, 85]. Consistent with the enhanced in vitro migration of miR-200-expressing 4TO7 cells, they produced more metastases from mammary tumors formed by injection into the mammary fat pad of BALB/cJ mice [86]. Clearly more work is required to resolve why miR-200 appears to inhibit invasion and metastasis in some systems but to apparently promote it in the 4T07 model.

Loss of E-cadherin is a key characteristic of EMT and is associated with tumour progression, metastasis and poorer prognosis in breast cancer [72, 87, 88]. Knockdown of E-cadherin in breast cancer cells is sufficient to dramatically increase metastasis when these cells are injected into nude mice [42]. In accordance with its ability to suppress E-cadherin, miR-9 can regulate metastasis [43]. However, the increased migration and invasion of miR-9 expressing HMLE or SUM149 cells in vitro can only partially be rescued by ectopic expression of E-cadherin, suggesting that miR-9 may have other mRNA targets that effect migration and invasion. Importantly, the link between miR-9 and metastasis is likely to be clinically relevant, because significantly higher miR-9 levels were observed in primary breast tumours from patients with metastases compared to patients with no metastases [43]. Since miR-9 levels are lower in metastases compared to matched primary tumour samples [77], miR-9 maybe involved in an early step in the metastatic process.

Suppression of the EMT-inducer, Twist, inhibits metastasis of breast cancer cells injected into the mammary gland of Balb/c mice [40]. A key target of Twist is miR-10b, as loss of miR-10b suppresses the Twist-induced migration and invasion of HMLE cells in vitro [41]. MiR-10b is capable of promoting invasion in vitro and metastasis upon orthotopic injection into NOD/SCID mice, as shown with over-expression of miR-10b in non-invasive, non-metastatic SUM149 breast cancer cells. MiR-10b suppresses homeobox D10 (HOXD10), thus permitting the expression of the pro-metastatic protein RHOC [41]. While it is clear that miR-10b can initiate metastasis, the correlation of miR-10b and breast cancer is complicated. miR-10b levels are reduced in breast cancer samples compared to normal tissue [75, 89] and miR-10b expression does not correlate with distant metastases, recurrence-free survival or distant relapse free survival [89]. To reconcile these observations, a likely hypothesis is that induction of EMT at the invasive front of a breast tumour may lead to transient activation of Twist and miR-10b, thus promoting invasion and metastasis [41, 90]. Further investigation is required to assess the precise role of miR-10b in metastasis and its link to EMT.

The pro-tumorigenic, pro-metastatic role of miR-21 has been firmly established [91]. Increased miR-21 expression is consistently observed in breast cancer, particularly invasive breast cancer [92–95] and high levels of miR-21 correlate with poor disease free survival and high levels of TGF-β1 [96]. MiR-21 directly suppresses known metastasis suppressors, including Tropomyosin 1 (TPM1), PDCD4 and maspin [91]. Further investigation is required to determine whether reversion of mesenchymal, metastatic cells to an epithelial phenotype would lead to a reduction in miR-21 and a suppression of metastasis. Manipulation of EMT, through the differential expression of these EMT-related miRNAs, may present a novel therapeutic strategy for the treatment of advanced breast cancer although many obstacles remain, with delivery being one of them.

## miRNAs, EMT and Breast Cancer Stem Cells

The concept of cancer stem cells is controversial and may not apply to all human cancers, but support for the cancer stem cell model comes from the ability to fractionate cancer cells into populations enriched for tumour-initiating cells. Breast cancer stem cells (bCSCs) can be enriched from solid breast tumours or pleural effusions from metastatic breast cancer patients according to cell surface markers ($CD44^{+}CD24^{-/low}$) and serially passaged in immunocompromised mice, generating tumours each time that have the same heterogeneous mix of cells present in the initial tumour, consistent with the notion of stem cell-like properties [97]. Aldehyde dehydrogenase 1 (ALDH1) activity is also used to enrich for bCSCs and is associated with metastatic ability and poor prognosis of breast cancer [98, 99]. These studies indicate that bCSC frequency varies depending on treatments, grade and sub-type of breast cancer. Regardless of the method of enrichment, bCSCs have increased tumorigenic potential and propensity to metastasise compared to other cancer cells [97, 99]. This has led to the generation of an "invasiveness" signature by expression profiling of $CD44^{+}CD24^{-/low}$ (bCSC-like) cells relative to normal breast epithelium, which is highly predictive of the propensity of a tumour to metastasise [100]. Importantly, bCSCs are resistant to current chemotherapy and radiation therapies [101–104]. The combination of the bCSC properties of self-renewal, ability to reconstitute a tumour and resistance to therapy facilitate recurrence in advanced breast cancer.

There is much evidence linking bCSCs and EMT. BCSCs enriched from breast tumours and metastatic breast pleural effusions express markers similar to cells that have undergone an EMT [105–107]. Similarly, EMT and stem cell markers are frequently associated with breast cancers that have a propensity to metastasise, such as basal-like [73] and metaplastic [108] breast cancers. Consistent with the expression of EMT markers in bCSCs, there is differential expression of EMT-related miRNAs in bCSCs compared to other breast cancer cells. miRNA expression profiling of bCSCs isolated from human breast tumours compared to the remaining breast cancer cells showed high levels of miR-155 and low levels of the miR-200 family in bCSCs [48]. In addition, low to undetectable levels of the miR-200 family are found in chemotherapy-resistant bCSCs isolated from breast cancer cell lines [104]. However, the most direct association between bCSCs and EMT comes from studies showing that the induction of EMT in vitro in transformed mammary epithelial cells [106, 109] or in vivo in epithelial breast cancer cells in mouse models [110] generates cells with bCSC properties. This suggests that through the EMT program breast epithelial cells can gain bCSC properties. Accordingly, the EMT program is involved in cancer progression. To assess whether reversal of EMT may suppress cancer stem cell properties, several groups have manipulated levels of regulators of EMT. For example, knockdown of ZEB in pancreatic cancer cell lines suppresses cancer stem cell properties [79]. Similarly, miR-200c expression in bCSCs suppresses cancer stem cell properties, as demonstrated by reduced tumorigenicity of miR-200c-expressing $CD44^{+}CD24^{-/low}$ cells (isolated from an early passage human breast xenograft tumour) [48]. This study showed that in addition to enforcing the epithelial phenotype through repression of ZEB, miR-200c acts upon self-renewal pathways through regulation of BMI1. Further miR-200 family targets have been identified that are involved in self-renewal and cancer stem cell properties, such as Sox2 and Klf4 [79]. Therefore, the miR-200 family is able to affect two key properties of cancer stem cells — differentiation and self-renewal.

## miRNAs that Affect Breast Cancer Response to Endocrine- or Chemotherapy

There are many determinants of sensitivity and resistance to cancer therapies such as drug metabolizing enzymes, drug transporters, proteins involved in DNA repair, cell division, and apoptosis, and levels of the drug targets themselves (such as estrogen receptor $\alpha$ for breast cancer endocrine therapy). MiRNAs can regulate these determinants and thereby influence sensitivity to therapeutic agents, as evidenced by knockdown of Dicer in breast cancer cells increasing sensitivity to cisplatin [111]. Furthermore, chronic exposure to anti-cancer agents can affect expression of miRNAs and lead to changes in the pharmacokinetic and pharmacodynamic properties of the agents.

The most global study to date on this topic examined the effects of three miRNAs, let7i, miR-16 and miR-21, which target RAS, BCL2, and PTEN respectively, and also used in silico methods to compare drug potency patterns of 3,089 compounds to miRNA expression profiles across the entire NCI-60 human cancer cell line panel [112]. The three miRNAs tested were capable of altering the potency of a number of anti-cancer agents by up to 4 fold and the in silico comparison of drug potencies to miRNA expression profiles across the NCI-60 panel demonstrated that 30 miRNAs, including miR-21, showed significant correlation with the potency of numerous anti-cancer agents, indicating a substantial role for miRNA in determining drug responsiveness [112]. Specific examples of miRNA affecting drug response by directly targeting important determinants of drug sensitivity or resistance are rapidly accumulating, including targeting of BCRP/ABCG2 and CYP1B1 by miR-328 and miR-27b [113, 114].

Increasing evidence links EMT and drug resistance. Epithelial markers are lost in cetuximab-resistant urothelial carcinoma cell lines [115], gemcitabine-resistant pancreatic cancer cells [116] and in erlotinib- and gefitinib-resistant non-small cell lung carcinoma and head and neck squamous cell carcinomas [117, 118], suggesting that these drug-resistant cells have undergone an EMT. Similarly, residual breast cancers after chemotherapy have low expression of E-cadherin and high expression of mesenchymal markers [119]. Additionally, EMT induced by knockdown of E-cadherin in HMLE cells increases resistance to doxorubicin, actinomycin D and paclitaxel [120].

Specifically linking the process of EMT with chemotherapy resistance is the finding that miR-200 not only represses ZEB1 and ZEB2, but also directly represses other mesenchymal and neuronal genes such as fibronectin, moesin, NTRK2 and class III beta-tubulin (TUBB3), not normally expressed in epithelial cells, but aberrantly expressed in high-grade, de-differentiated carcinoma cells that have undergone EMT [81]. Expression of TUBB3 (an isoform of tubulin normally limited to neuronal cells) is a common mechanism of resistance to microtubule-binding chemotherapeutic agents in many types of carcinoma, including breast cancer [121–124]. Enhanced miR-200c expression in carcinoma cells dramatically increases sensitivity to microtubule-targeting agents [81]. The ability of miR-200c to restore chemosensitivity to taxanes is attributable to its ability to directly target TUBB3 since introduction of exogenous non-targetable TUBB3 lacking its 3'UTR miR-200c target site, reverses the effect [125]. Similarly, miR-200b and miR-200c can increase the sensitivity of breast cancer cells to doxorubicin in vitro [85]. Additionally, miR-205 suppresses ERBB3/HER3 (a ligand binding, kinase inactive receptor tyrosine kinase of the epidermal growth factor receptor (EGFR) family). MiR-205 expression increases the sensitivity of breast cancer cells to the epidermal growth factor receptor inhibitors, gefitinib and lapatinib [52].

Nearly, 70% of breast cancers express estrogen receptor alpha (ESR1) and are consequent candidates for endocrine therapy. Tamoxifen, is the most commonly prescribed endocrine therapy for pre-menopausal women (while aromatase inhibitors are commonly used in post-menopausal women) and tamoxifen has also been recommended as a preventative. Nevertheless, 30–40% of patients with ESR1-positive tumours fail adjuvant tamoxifen therapy and nearly all patients with metastatic disease develop tamoxifen resistance [126–128]. De novo and acquired tumor resistance to endocrine therapy remains a poorly understood clinical problem. Recent studies have identified multiple miRNAs that target ER and affect ER-signalling [22, 86, 129–136]. MiR-206 has been demonstrated to directly target ESR1 [135] and may modulate tamoxifen resistance [130] and this miRNA appears to be up-regulated in ESR1-negative tumours [75]. However, the most compelling evidence of the critical role of miRNAs in downregulating ER and contributing to the acquisition of tamoxifen resistance has emerged from studies of miR-221/222, which are highly expressed in ESR1 negative breast tumours and cells lines, directly target ER$\alpha$ and render cells resistant to tamoxifen [132, 133]. While additional miRNAs differentially expressed in tamoxifen-resistant cell lines have been identified, their functional role in tamoxifen-resistance has not been elucidated [133].

## Conclusions

Manipulation of EMT-related miRNAs may represent a novel therapeutic strategy for the treatment of advanced breast cancer. The attractiveness of using miRNAs as targeted therapeutics arises from the fact that unlike traditional gene therapy, the miRNA can simultaneously target many key genes/proteins involved in the process of EMT. As an example, expression of the differentiation- and "stemness"-associated miR-200 or miR-203 cannot only suppress transcription factors that repress E-cadherin, but they also target genes normally only expressed in mesenchymal cells or stem cells. Some of these targets may only be elucidated following proteomic analysis. In theory EMT-related miRNAs hold potential as a form of "differentiation therapy" that would drive differentiation, reduce invasion, and enhance chemosensitivity. However, in reality many obstacles remain, with delivery and timing being the largest hurdles to overcome. It remains to be determined how miRNAs introduced to prevent or reverse EMT in breast carcinoma cells will affect normal differentiated epithelium or normal stem cells. Furthermore, recent findings raise the question as to whether it will be detrimental to drive an MET in cancer [137].

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